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Identification of Hexose Transporter-Like Sensor *HXS1* and Functional Hexose Transporter *HXT1* in the Methylotrophic Yeast *Hansenula polymorpha*[▽]

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We identified in the methylotrophic yeast *Hansenula polymorpha* (syn. *Pichia angusta*) a novel hexose transporter homologue gene, *HXS1* (*h*ex*s*ose *s*ensor), involved in transcriptional regulation in response to hexoses, and a regular hexose carrier gene, *HXT1* (*h*ex*t*ransporter). The Hxs1 protein exhibits the highest degree of primary sequence similarity to the *Saccharomyces cerevisiae* transporter-like glucose sensors, Snf3 and Rgt2. When heterologously overexpressed in an *S. cerevisiae* hexose transporter-less mutant, Hxt1, but not Hxs1, restores growth on glucose or fructose, suggesting that Hxs1 is nonfunctional as a carrier. In its native host, *HXS1* is expressed at moderately low level and is required for glucose induction of the *H. polymorpha* functional low-affinity glucose transporter Hxt1. Similarly to other yeast sensors, one conserved amino acid substitution in the Hxs1 sequence (R203K) converts the protein into a constitutively signaling form and the C-terminal region of Hxs1 is essential for its function in hexose sensing. Hxs1 is not required for glucose repression or catabolite inactivation that involves autophagic degradation of peroxisomes. However, *HXS1* deficiency leads to significantly impaired transient transcriptional repression in response to fructose, probably due to the stronger defect in transport of this hexose in the *hxs1*Δ deletion strain. Our combined results suggest that in the Crabtree-negative yeast *H. polymorpha*, the single transporter-like sensor Hxs1 mediates signaling in the hexose induction pathway, whereas the rate of hexose uptake affects the strength of catabolite repression.

As a favorite carbon substrate, glucose exerts numerous strong and well-coordinated effects on the physiological state of yeast cells. They include gene-specific regulation of transcription and mRNA stability as well as regulation at the post-translational level: e.g., catabolite inactivation of certain glucose-repressible enzymes (3, 13, 19). Signaling pathways involved in different glucose effects have been studied mostly in the model yeast *Saccharomyces cerevisiae*. A number of participating components have been identified, and some have been shown to have conserved functions in other yeast species (for review, see references 12, 36, and 41). Nevertheless, knowledge of glucose-triggered regulatory pathways for Crabtree-negative yeasts (which contrary to *S. cerevisiae*, are unable to produce ethanol aerobically in the presence of high external glucose concentrations) (11) that primarily rely on respiratory metabolism still remains very limited. In particular, the first stages, how glucose is sensed, and the molecular triggers involved in different pathways are the least understood aspects of these yeast species.

Two nontransporting glucose carrier homologues, Snf3 and Rgt2, have been shown to function as glucose sensors in *S. cerevisiae* in a pathway of transcriptional induction. They differentially regulate expression of the functional hexose transporters in response to extracellular glucose concentration (32). This mechanism provides fast adaptation of the hexose transport system to glucose availability. Recent studies revealed that the *S. cerevisiae* high-affinity glucose sensor ScSnf3 and the low-affinity sensor ScRgt2 physically interact with membrane-associated Yck1 casein kinase I, which transduces the signal further downstream to Mth1/Std1, and Rgt1, which directly regulates expression of the target genes (16, 29). Apart from *S. cerevisiae*, transporter-like glucose sensors with orthologous functions have been described in other yeasts: *Kluyveromyces lactis* (KlRag4) (4), and *Candida albicans* (CaHgt4) (5). It was shown that the glucose-induced signal transduction requires the C-terminal cytoplasmic fragment of these sensors. In ScSnf3, ScRgt2, and KlRag4, this protein region harbors a so-called "glucose sensor domain," a 25-amino-acid-long conserved sequence apparently involved in interaction with Yck1 (4, 29, 32, 48). In CaHgt4, however, this motif is missing or at least degenerate (5).

It is generally accepted that, contrary to the glucose induction mechanism, glucose must enter the cell to trigger the repression pathway (4, 51). While catabolite repression is manifested to different extents in all yeast species, the molecular mechanisms involved and the target genes are not necessarily the same (45). The key elements of the main glucose repression pathway in baker's yeast include the Snf1 kinase complex, the Reg1/Glc7 phosphatase complex, Mig1, and related transcriptional repressors in conjunction with general Tup1/Ssn6 repressor complex (12).

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The glycolytic enzyme hexokinase is a good candidate for the intracellular glucose sensor as glucose acts as its substrate. It was shown that hexokinase is required for repression in several yeasts (12). In *S. cerevisiae*, hexokinase II has a regulatory role in repression different from its enzymatic function, suppressing at high glucose the nuclear localization of Mig1 by blocking its Snf1-mediated phosphorylation (1). In *H. polymorpha* (syn. *Pichia angusta*) (22), an object of this study, hexose phosphorylation activity, either via hexo- or glucokinase, was demonstrated to be essential for catabolite repression (20).

Elements of the glucose transport system, in principle, could also mediate a sensing function for repression. Such sensing, if it exists, is expected to depend on the mode by which glucose is taken up in different yeasts (active transport versus facilitated diffusion). An example is known from the fungus *Neurospora crassa*, where deficiency in transporter-like sensor Rco3 apparently directly affects glucose repression through a yet unknown mechanism (28). In *S. cerevisiae*, however, none of its main hexose facilitators has been directly implicated in the repression mechanism (35) and repression signaling was found to be independent of plasma membrane sensors (3). In certain cases, as in the *K. lactis rag4* mutant, deficiency in induction of glucose transporters leads to insensitivity to glucose repression, but this mainly occurs due to the "effector exclusion" resulting from the impaired glucose uptake (4).

Little is known about the glucose-sensing and -signaling mechanisms involved in catabolite inactivation. In *S. cerevisiae*, glucose signaling for catabolite inactivation of the cytosolic gluconeogenic enzyme fructose-1,6-bisphosphatase is independent of transporter-like glucose sensors or hexokinase II but is mediated, at least in part, by Gpr1, a glucose-sucrose binding G protein-coupled receptor (GPCR) (3, 26). The role of glucose transport in supporting this pathway remains unclear. For peroxisomal enzymes, catabolite inactivation involves degradation of superfluous peroxisomes in vacuoles via the process of pexophagy, best studied in methylotrophic yeasts (for review, see reference 9). In the yeast *Pichia pastoris*, a subunit of the glycolytic enzyme phosphofructokinase was demonstrated to be required for glucose signal transduction in pexophagy and this function was independent of its catalytic activity (52). Recently, the Gpr1 sensor of the cyclic AMP-dependent pathway was implicated in glucose-induced pexophagy in *S. cerevisiae* (30).

The methylotrophic yeast *H. polymorpha* has been a very useful organism in studies of the molecular mechanisms of peroxisome biogenesis and degradation, both regulated by glucose (24). Also, *H. polymropha* is a biotechnologically important yeast known as an efficient expression platform for heterologous proteins, governed mostly by glucose-repressible promoters (14, 21). In addition, this thermotolerant yeast has recently been suggested as a promising organism for high-temperature fermentation of major sugars of lignocellulose hydrolysates, glucose and xylose (38). Therefore, comprehensive knowledge of the glucose-triggered pathways in this yeast is required to optimize the *H. polymorpha* expression platform or to construct strains capable of simultaneous utilization of glucose and other sugars derived from lignocellulose. Taking into account the availability of the *Hansenula polymorpha* full genome sequence (34) and developed versatile molecular techniques, this yeast may further serve as a convenient model for elucidating glucose-sensing pathways in lower eukaryotes (20, 45, 46).

We have previously demonstrated in *H. polymorpha*, that the Gcr1 protein, which is similar to glucose sensors, is required for efficient glucose transport and glucose repression but not for pexophagy (46). While expression of genes of methanol metabolism in *H. polymropha* is strictly repressed in the presence of hexoses, disaccharides, and ethanol, only glucose repression (and, to a lesser extent, repression triggered by fructose and mannose) is dependent on Gcr1 (46). Gcr1 lacks the C-terminal sequence extension typical for a number of sensors and required for signaling. Its mode of action (transport versus sensing) remains to be elucidated.

We screened the *H. polymorpha* full-genome-sequence database (34) for the presence of other hexose transporter homologues with possible sensing function in glucose regulation of gene expression. In this report, we present a functional analysis of Hxs1, a novel orthologue of *S. cerevisiae* Snf3 and Rgt2 nontransporting sensors involved in signaling for transcriptional induction, and Hxt1, the first functional hexose transporter identified in *H. polymorpha*, which is regulated by Hxs1.

MATERIALS AND METHODS

Strains, media, and microbial techniques. The *H. polymorpha* and *S. cerevisiae* strains used are listed in Table 1. The *tup1*Δ mutant (25) was kindly provided by Ida van der Klei (University of Groningen, The Netherlands). The cells were cultivated on standard liquid or solid media at 37°C as described previously (45). The concentration of each of the carbon sources was 1% (wt/vol or vol//vol), unless indicated otherwise. Cell density was determined by absorbance at 600 nm. Yeast transformation by electroporation was performed as described previously (10). Cultivation of *Escherichia coli* DH5α and standard recombinant DNA techniques were performed essentially as described previously (40).

Plasmid and strain construction. (i) *HXS1* deletion strain. A vector capable of deleting most of the Hp*HXS1* open reading frame (ORF) was constructed in two steps. As the first step, a 1.2-kb fragment of *H. polymorpha* genomic DNA, composed of sequences from the 3′ terminus of *HXS1*, beginning at nucleotide 1015 of the corresponding ORF and adjacent 3′-flanking region of approximately 0.3 kb was amplified by PCR using *H. polymorpha* genomic DNA as a template with *Taq* DNA polymerase (Fermentas, Lithuania). The primers for this PCR, OL74 and OL75 (Table 2), included restriction sites for PstI and HindIII, respectively. The fragment was cloned into PstI/HindIII-digested plasmid pYT1 (47) carrying the Sc*LEU2* gene as a selectable marker, to create vector pOH10. As the next step, a 0.85-kb fragment containing sequences just 5′ of the methionine initiator ATG of *HXS1* was PCR amplified from genomic DNA with primers OL72 and OL73, which included restriction sites for BamHI and XbaI, respectively. The 5′-flanking fragment was inserted into BamHI/XbaI-digested plasmid pOH10 to create pOH11. The latter plasmid was digested with HindIII, releasing a 3.9-kb fragment comprised of Sc*LEU2* flanked by *HXS1* 5′ and 3′ sequences and transformed into strain NCYC 495 *leu1-1* by electroporation. Several hundred prototrophic transformants were selected and analyzed for possible deficiency in growth on hexoses. To confirm deletion of the *HXS1* gene in several clones exhibiting slow growth on fructose, genomic DNAs were isolated and used as templates in PCRs with two sets of oligonucleotide primers. One set was composed of primer OL72, complementary to sequences in the 5′ flanking region of the *HXS1* ORF, and primer OL75, which hybridized to sequences in the 3′ region of the *HXS1* ORF. With this set of primers, genomic DNA from the wild-type strain yielded a fragment of 3.09 kb in size, and DNAs from potential *hxs1*-knockout strains or plasmid pOH11 produced a fragment of 3.90 kb. Another set of primers contained the same 3′ flanking sequence primer OL75 and a second primer, CK15, complementary to a sequence in Sc*LEU2*. It did not hybridize with the wild-type genomic DNA but produced a 1.3-kb-long fragment with genomic DNAs from potential knockout strains or pOH11 as templates. Correct insertion of the *HXS1* deletion cassette was further confirmed via Southern blotting by hybridizing HindIII-cut total DNAs of the wild-type strain and of several potential *hxs1*-knockout mutants with the Sc*LEU2* fragment isolated as a 1.4-kb PCR product with primers K22 and KD21 and plasmid pYT1

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference
H. polymorpha		
NCYC 495	*leu1-1*	10
*gcr1*Δ	*gcr1*Δ::Sc*LEU2 leu1-1met6*	46
*hxs1*Δ	*hxs1*Δ::Sc*LEU2 leu1-1*	This study
*hxs1*Δ/Hxs1^{R203K}	*hxs1*Δ::Sc*LEU2leu1-1*; pOH13 (pGLG578 + Hp*HXS1-1*)	This study
*hxs1*Δ/Hxs1Δ*C38*	*hxs1*Δ::Sc*LEU2 leu1-1*; pOH17 (pPICZB + Hp*HXS1*Δ*1*)	This study
*hxs1*Δ/Hxs1Δ*C95*	*hxs1*Δ::*ScLEU2 leu1-1*; pOH18 (pPICZB + Hp*HXS1*Δ*2*)	This study
*hxs1*Δ/Hxs1^{R203K}Δ*C95*	*hxs1*Δ::Sc*LEU2 leu1-1*; pOH19 (pPICZB + Hp*HXS1-1*Δ*2*)	This study
*tup1*Δ	*tup1*Δ::Hp*URA3 ura3 leu1-1*	25
*hxt1*Δ	*hxt1*Δ::Sc*LEU2 leu1-1*	This study
S. cerevisiae		
VW1A	*MAT*α *leu2-3,112 ura3-52 trp1-289 his3*Δ*1 MAL2-8C SUC2*	50
EBY.VW4000[a]	*leu2-3,112 ura3-52 trp1-289 his3*Δ*1 MAL2-8C SUC2 hxt17*Δ *hxt13*Δ *hxt15*Δ *hxt16*Δ *hxt14*Δ *hxt12*Δ *hxt9*Δ *hxt11*Δ *hxt10*Δ *hxt8*Δ *hxt514*Δ *hxt2*Δ *hxt367*Δ *gal2*Δ *slt1*Δ *agt1*Δ *ydl247*Δ *yjr160c*Δ	50
EBY.VW4000/HpGcr1	EBY.VW4000/pOH20 (pBM2974 + Hp*GCR1*)	This study
EBY.VW4000/HpHxs1	EBY.VW4000/pOH15 (pBM2974 + Hp*HXS1*)	This study
EBY.VW4000/HpHxt1	EBY.VW4000/pOH16 (pBM2974 + Hp*HXT1*)	This study
EBY.VW4000/ScSnf3	EBY.VW4000/pBM3135 (pBM2974 + Sc*SNF3*)	32
EBY.VW4000/ScHxt1	EBY.VW4000/pBM3362 (pBM2974 + Sc*HXT1*)	32

[a] *hxt*-null mutant.

(47) as a template. One transformant, *hxs1*Δ::Sc*LEU2 leu1-1*, was utilized throughout this study as an *hxs1* deletion strain (*hxs1*Δ).

(ii) Hxs1-mutated alleles. The Hp*HXS1-1* allele encoding the mutated gene with an R203K substitution was constructed as follows. First, two fragments of *HXS1-1* were amplified in PCRs with primers OL178 and OL179 (includes 1,217 kb of upstream promoter sequence and the 5′ part of the *HXS1* ORF) and primers OL180 and OL181 (consists of the 3′ part of the ORF and 194 bp of downstream flanking terminator sequence), using the High-Fidelity polymerase kit (Fermentas, Lithuania). The two fragments were then used as templates in PCR with flanking primers OL178 and OL181 to produce the full-length *HXS1-1* gene that contains the OL179 and OL180 desired mutation (Table 2). The resulting *HXS1-1* allelle was treated with BamHI and NotI and cloned into vector pGLG578 (44) cut with the same enzymes. The presence of the correct R203K substitution in the resulting vector pOH13 was verified by sequencing. pOH13 was linearized with BamHI prior to transformation into the *hxs1*Δ recipient strain, and geneticin-resistant colonies were selected. Correct integration of *HXS1-1* into the genome in selected transformants was confirmed by PCR with primers OL178 and OL181.

The *HXS1*Δ*1*, *HXS1*Δ*2*, and *HXS1-1*Δ*2* alleles coding truncated forms of Hxs1—Hxs1Δ*C38*, Hxs1Δ*C95*, and Hxs1^{R203K}Δ*C95*, lacking 38 or 95 C-terminal amino acids, respectively—were amplified by PCR with primer pairs OL72 and OG3 (for *HXS1*Δ*2* and *HXS1-1*Δ*2*) and OL72 and OG4 (for *HXS1*Δ*1*). Amplified fragments were digested by BamHI and EcoRI and cloned into plasmid pPICZB (Invitrogen), treated with BglII and EcoRI. The resulting plasmids pOH17 (*HXS1*Δ*1*), pOH18 (*HXS1*Δ*2*), and OH19 (*HXS1-1*Δ*2*) were linearized with HindIII prior to transformation into an *hxs1*Δ recipient strain, and zeocin-resistant colonies were selected. The presence of *HXS1*Δ*1*, *HXS1*Δ*2*, and *HXS1-1*Δ*2* fragments in the genome of several isolated transformants was confirmed by PCR with primer pairs OL72 and OG3 and OL72 and OG4.

(iii) Heterologous complementation. The plasmids pOH15 and pOH16, which express *H. polymorpha* genes *HXS1* and *HXT1*, respectively, from the *ADH1* promoter in the multicopy plasmid pBM2974 (32) (Table 2), were created. The *HXS1* coding region was amplified as a 1.92-kb HindIII-EcoRI fragment with primers OG1 and OG2; the *HXT1* coding region was amplified as a 1.70-kb HindIII-SacI-flanked fragment with primers OG5 and OG6. The two fragments were cloned into corresponding sites of pBM2974. The constructed plasmids (pOH15 and pOH16) and control plasmids pBM3135 and pBM3362 (Table 1; kindly provided by M. Johnston [St. Louis, MO]) were transformed into *S. cerevisiae* strain EBY.VW4000 (Table 1), deleted for all hexose transporters and, therefore, unable to grow on glucose or fructose (kindly provided by E. Boles, Dusseldorf University, Germany). Transformants were selected as prototrophs for uracil on ethanol-containing plates and further analyzed for the ability to utilize hexoses.

Northern blotting. Northern blot analysis was performed essentially as described previously (40). For RNA preparation, cultures of *H. polymorpha* cells grown to mid-logarithmic phase in YPE medium (1% yeast extract, 2% peptone, and 1% ethanol) were washed with water and resuspended in mineral YNB medium containing 1% glucose or 1% methanol. Cells were harvested at the time points 20, 40, 60, and 80 min. Total RNA samples were prepared using the TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. Substrates for the probes specific to *HXS1*, *HXT1*, and *ACT1* were amplified by PCR using *H. polymorpha* genomic DNA as the template and the primer pairs HP39F and HP40R, HP43F and HP44R, and HP41F and HP42R, respectively. Probes were generated by random labeling with radioactive dCTP using a High Prime kit (Roche Diagnostics Corporation).

Q-PCR. For quantitative PCR (Q-PCR), *H. polymorpha* cells grown to log phase in rich YPE medium with 1% (vol/vol) ethanol were diluted to an optical density at 600 nm (OD_{600}) of 5.0 in synthetic mineral medium (YNB) with selected carbon sources and incubated for 30, 60, and 120 min at 37°C. Total RNA samples were prepared using the TRIzol reagent (Invitrogen) and treated with DNase (Fermentas, Lithuania), and 1 μg was used in a 20-μl reverse transcription reaction with the First Strand cDNA synthesis kit (Fermentas, Lithuania). Ten nanograms of cDNA was used in each 25-μl PCR probe. Primers for genes undergoing this analysis were designed using software from ABI (ABI PRISM Primer Express). A typical experiment was designed with the ABI 2× SYBR green PCR master mix (ABI no. 4309155). The comparative threshold cycle (C_T) method was used for analyzing data obtained from real-time PCR, essentially as described previously (33). The amount of target (under repression/induction condition) was normalized to an endogenous reference gene, Hp*ACT1*, and relativized to the control sample (derepression condition).

Sugar uptake assays. For glucose/fructose transport assays, cells were pregrown until the mid-logarithmic phase on YPE medium and then shifted to YNB medium supplemented with different concentrations of hexoses, as indicated in the figure legends. After preincubation for 2 h in YNB with different levels of glucose, 50 μl of labeled glucose/fructose with different concentrations of unlabeled sugar and prewarmed to 37°C was added to 100 μl of cells preincubated in a conical test tube at 37°C for 2 min, and exactly after 5 s the reaction was terminated by the addition of 10 ml of ice-cold 100 mM K phosphate buffer (pH 6.5) containing 500 mM unlabeled glucose/fructose. The cells were immediately filtered onto a glass fiber filter under reduced pressure and washed on the filter with 10 ml of ice-cold 100 mM K phosphate buffer (pH 6.5). The filters were then transferred into scintillation vials containing 5 ml of scintillant, and radioactivity was measured with a Beckman liquid scintillation counter.

Biochemical methods. All biochemical methods for preparation of crude cell extracts, measurement of protein concentration, determination of peroxisomal alcohol oxidase (AO) specific activity in cell extracts, and Western blot analysis

TABLE 2. Primers used in this study

Method and gene	Primer name	Sequence
Molecular cloning		
Hp*HXS1*	OL72	5′-TGTGGATCCAAAGCTTAAGGAGACGCT-3′
	OL73	5′-GGTTCTAGATAATATGGGCACAGGGA-3′
	OL74	5′-TAACTGCAGCAGTGCTCGGGAATTAAT-3′
	OL75	5′-TGGAAGCTTCCTATGATCAACATCTAC-3′
	OG1	5′-TGGAAGCTTATGTCGACAGAAGCTCGAGA-3′
	OG2	5′-GGTGAATTCTTATTGTCCATGGCTATGT-3′
	OG3	5′-GGTGAATTCTTACTTGAAATACATGGAC-3′
	OG4	5′-GGTGAATTCTTAGTATTCGCTCAGGTGC-3′
	OL178	5′-TGTGGATCCTCAGCTCTTACAAGACCATC-3′
	OL179	5′-CAGATCCTTTGATCCACTTGGGCGACA-3′
	OL180	5′-GGATCAAAGGATCTGTGATTTCGTTTTACC-3′
	OL181	5′-TATGCGGCCGCTCGGTGGTCTTCATTT-3′
Hp*HXT1*	OG5	5′-AACAAGCTTATGTCTAACGCGGATCAA-3′
	OG6	5′-GAAGAGCTCTTTATCTATCAGTCAATAA-3′
	OL80	5′-AACCAATTGATGTCTAACGCGGATCA-3′
	OL108	5′-TGTGGATCCGAATTTACTCTTTGGTTC-3′
	OL109	5′-GGTTCTAGAGAGAATAAAACTAGGGAG-3′
	OL110	5′-TAACTGCAGATCCAAACCAGAGTGTG-3′
	OL111	5′-TGGAAGCTTAAATGCCTGCAAGCGAG-3′
Sc*LEU2*	CK15	5′-TGTAATTGTTGGGATTCC-3′
	K22	5′-TGCTCTAGAAGGTGGTTAGCAATCGTC-3′
	KD21	5′-CTAGTCTAGAGTGTGGTGCCCTCCTCCTTG-3′
Northern blot		
Hp*HXS1*	HP39F	5′-ATGCTCACAGGCATGCTT-3′
	HP40R	5′-ATAGCTCGTCCACCTCCT-3′
Hp*HXT1*	HP43F	5′-AATCCTTGCAGCAACTGT-3′
	HP44R	5′-TGTAGTCGATGTACAGCT-3′
Hp*ACT1*	HP41F	5′-AGATACCCAATTGAGCAC-3′
	HP42R	5′-CATTCTCTCAGCAATACC-3′
Q-PCR		
Hp*HXS1*	F-*HXS1*	5′-CGAGCTATTCAGGGTGTGCAA-3′
	R-*HXS1*	5′-CCTCGTTCTCCTGCGTTTCT-3′
Hp*HXT1*	F-*HXT1*	5′-GTATCGGTATGGCCGTGTGTT-3′
	R-*HXT1*	5′-CCAAACTCGCCCTTGTACAGA-3′
Hp*ACT1*	F-*ACT1*	5′-TGTCGTCCCAGTTGGTAACG-3′
	R-*ACT1*	5′-GGCCCAATCCAAGAGAGGTAT-3′

were essentially performed as described previously (15, 46). Visualization of AO activity in yeast colonies was performed essentially as described by us earlier (43), with some modifications. Cells grown on YNB medium with different carbon sources were overlaid with 9 ml of agarized AO assay reaction mixture: 100 mM K phosphate buffer (pH 7.0), 0.3% (wt/vol) agarose, 0.05% (wt/vol) chromogen *o*-dianizidine, 0.5% (wt/vol) cetyltrimethylammonium bromide (CTAB) as a permeabilizing agent, 1% (wt/vol) methanol, and 3 U/ml of horseradish peroxidase. To prepare the mixture, first three components of the reaction mixture were briefly boiled and then CTAB was added and the solution was vigorously mixed. Upon cooling to 40°C, methanol and peroxidase were sequentially supplemented. Yeast colonies overlaid with the AO reaction mixture were incubated at 37°C for 0.5 to 1 h. High AO activity results in cells stained red.

Sequence analyses. Sequence alignments and phylogenetic analyses were performed using the ClustalW, version 1.6, algorithm (7). The BLAST Network Service of the National Center for Biotechnology Information (Bethesda, MD) at http://www.ncbi.nlm.nih.gov/BLAST/ was used to search for amino acid sequence similarities. Prediction of Hxs1 secondary structure was made using TMpred program from the http://www.ch.embnet.org/software/TMPRED_form.html server. For pattern and profile searches, the PROSCAN program at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_server.html was used (6).

Nucleotide sequence accesssion number. The sequences of the genes described in this report have been deposited in GenBank under accession no. EU476006 (Hp*HXS1*) and EU476007 (Hp*HXT1*).

RESULTS

Identification and sequence analysis of *H. polymorpha* Hxs1 and Hxt1 proteins. The protein product of the *H. polymorpha HXS1* (*h*exose *s*ensor) gene (contig 26, orf.375, *H. polymorpha* genome database; https://ssl.biomax.de/rheinbiotech) (34) was identified via database BLAST service as the closest intraspecies homologue of the previously described *H. polymorpha* Gcr1 (46). The deduced protein Hxs1 shares with Gcr1 44% and 62% of primary amino acid sequence identity and similar-

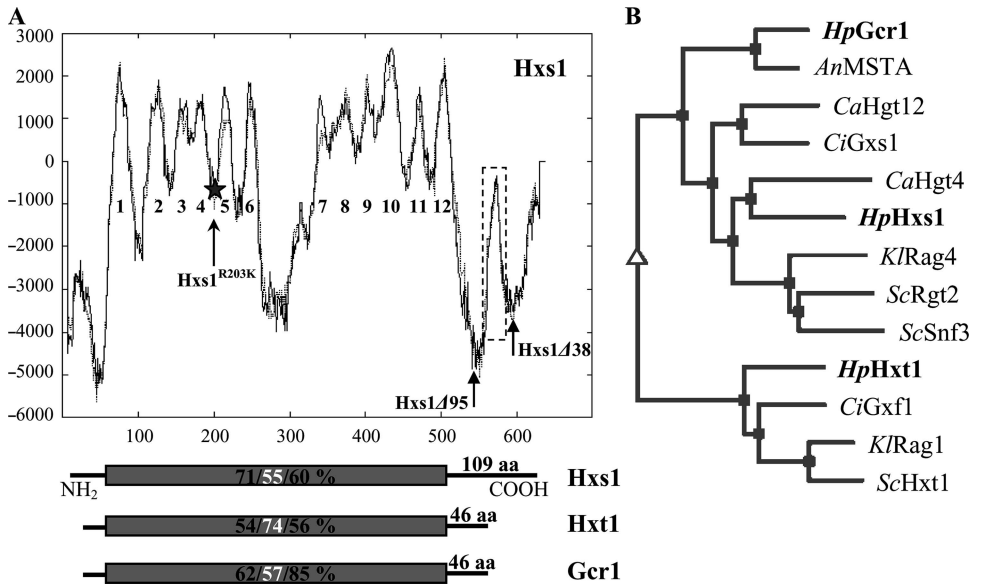

FIG. 1. (A)Predicted topology of the deduced Hxs1 amino acid sequence. Analysis was conducted using the TMpred program (see Materials and Methods) with a TM helix length between 17 and 33 residues. Hydropathy values are on the *y* axis, and the residue numbers are on the *x* axis. The 12 predicted membrane-spanning segments (TM 1 to 12) are numbered. Below is shown a schematic representation of three *H. polymorpha* hexose transporter homologues; from left to right, their percent core sequence similarity to ScSnf3, ScHxt1, and AnMSTA is indicated. The R203 residue in Hxs1 that, when mutated to K, converts Hxs1 into constitutively signaling form is denoted with a star. Vertical arrows indicate the size of constructed nonfunctional Hxs1 forms with portions of the C-terminal region deleted (Fig. 8). The dotted box indicates Hxs1 C-terminal fragment, similar in its hydropathy profile to the fragment that contains the "glucose sensor domain" in the other yeast sensors. (B) Phylogenetic tree based on primary sequence similarity depicting predicted evolutionary relationship of *H. polymorpha* Hxs1, Hxt1, and Gcr1 proteins to other yeast and fungal hexose transporters. The tree was built using the ClustalW algorithm for multiple alignments (6). To simplify the output format, only selected representatives of fungal transporters, whose function was experimentally studied, are depicted. The tree root is shown as an open triangle, and subtrees' roots are shown as solid squares. HpHxs1, HpHxt1, and HpGcr1 are highlighted in boldface. Species-specific abbreviations for each gene name are as follows: An, *Aspergillus niger*; Ca, *Candida albicans*; Ci, *Candida intermedia*; Hp, *Hansenula polymorpha*; Kl, *Kluyveromyces lactis*; and Sc, *Saccharomyces cerevisiae*; The GenBank accession numbers of the sequences are as follows: AnMSTA, AAL89822; CaHgt4, XP_723173; CaHgt12, XP_888662; CiGxs1, CAI44932; CiGxf1, CAI77652; HpGcr1, AAR88143; HpHxt1, EU476006; HpHxs1, EU476007; KlRag1, XP_453656; KlRag4, CAA75114; ScHxt1, M82963; ScSnf3, NP_010087; and ScRgt2, NP_010143.

ity, respectively, in their core 12 transmembrane (TM) regions, characteristic of all hexose transporters and other carriers in the major facilitator superfamily (39) (Fig. 1A). Hxs1 is 638 amino acid residues long and, contrary to Gcr1 which is 541 amino acid residues in length, possesses well-pronounced N- and C-terminal sequence extensions of 67 and 109 amino acids, respectively (Fig. 1A). Comparison with the available computer databases and phylogenetic analysis revealed that Hxs1 is a member of a distinct subgroup of hexose transporters that contains all other known yeast transporter-like sensors, including *S. cerevisiae* Snf3 and Rgt2 (Fig. 1B). Sequence comparison with its closest homologues demonstrated that Hxs1, similarly to the recently described *C. albicans* glucose sensor Hgt4 (5), does not contain the so-called "glucose-sensor domain" in its C-terminal region, characteristic of *S. cerevisiae* Snf3 and Rgt2 and *K. lactis* Rag4 and essential for their sensing function (4, 32, 48) (see Fig. S1 at http://www.cellbiol.lviv.ua/signal/articles/ec_hphxs1_supplement.pdf). Importantly, the C-terminal extension of Hxs1, when a BLAST search against the protein database was performed, and the ScSnf3 "glucose sensor" domain, when a BLAST search against the *H. polymorpha* genome database was performed, produced no clear hits.

It is noteworthy that Hxs1 appeared to be a closer homologue, compared to Gcr1, of yeast nontransporting sensors (54% and 53% identity to Snf3 and Rgt2, respectively, relative to 42% and 45% exhibited by the TM region of Gcr1). Therefore, by primary sequence similarity in a core region, Hxs1 is more closely related to glucose sensors from other yeasts than it is to Gcr1, suggesting *HXS1* and *GCR1* are not the result of gene duplication in the *H. polymorpha* genome. In fact, the closest Gcr1 homologues were found in filamentous fungi. The recently described *Aspergillus niger* MSTA (exhibiting 74% sequence identity to Gcr1) (49) and related yeast *Candida intermedia* Gxs1 (23) and *C. albicans* Hgt12 (27) (Fig. 1B) were all demonstrated to be high-affinity glucose symporters able to complement the glucose growth deficiency of the *S. cerevisiae* *hxt*-null (transporterless) strain, suggesting their functionality as glucose carriers.

We also observed that the *H. polymorpha* genome harbors multiple other hexose transporter-like genes whose predicted protein products exhibit less than 30% sequence similarity to Hxs1 and Gcr1. One noticeable exception is a product of the gene designated as *HXT1* (*h*exose *t*ransporter) (contig 6, orf.206). Hxt1 exhibits 37% and 34% amino acid sequence identity to Gcr1 and Hxs1, respectively, but its similarity to functional hexose transporters from other yeasts is significantly higher (e.g., approximately 60% identity to *S. cerevisiae* Hxt3 and *K. lactis* Rag1) (see Fig. S2 at http://www.cellbiol.lviv.ua/signal/articles/ec_hphxs1_supplement.pdf). Accordingly, Hxt1 falls into a phylogenetic clade clearly different from that of

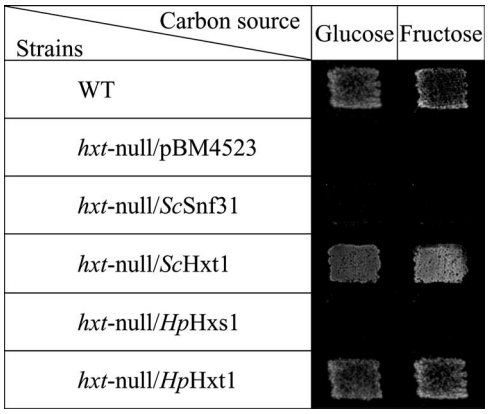

FIG. 2. Functional analysis of *H. polymorpha* hexose transporter homologues heterologously expressed in *S. cerevisiae*. Strain VW1A (Table 1) served as a wild-type (WT) control. The isogenic *hxt*-null mutant was transformed with empty vector pBM4523 or vectors expressing *S. cerevisiae* and *H. polymorpha* hexose transporter genes under the Sc*ADH1* promoter (see Materials and Methods for details). Cells of the transformants were grown for 2 days on solid media supplemented with different concentrations of glucose or fructose. (An example is shown with 2% [wt/vol].) Only vectors expressing Sc*HXT1* or Hp*HXT1* complemented the growth deficiency of the *S. cerevisiae* *hxt*-null strain on hexoses.

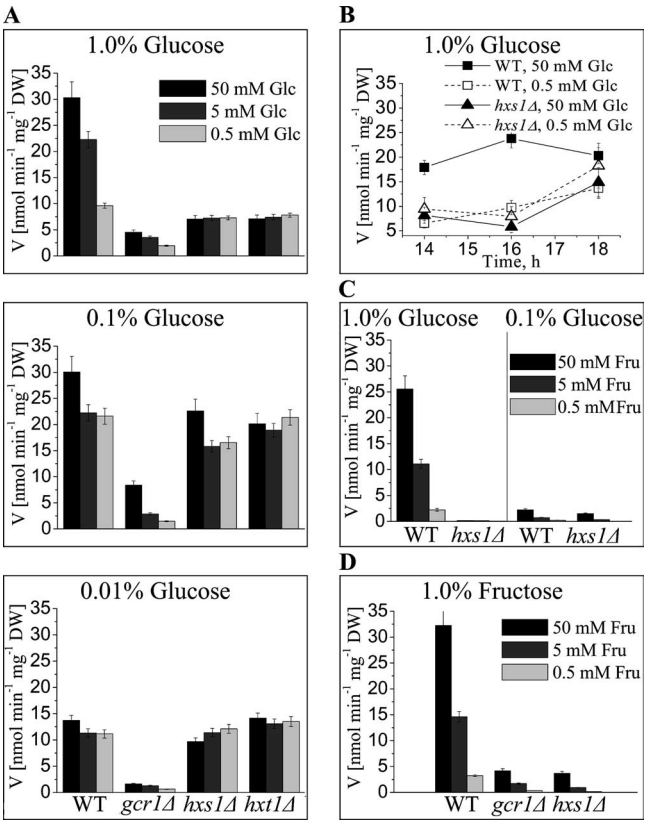

FIG. 3. *hxs1*, *hxt1*, and *gcr1* mutations differentially affect hexose transport. (A) Glucose (Glc) transport measured with different glucose levels (see further) after preincubation of the cells for 2 h with the indicated concentrations of glucose (%). Cells were grown to the mid-log phase (OD_{600} of 5.0) in liquid YPE medium and transferred to fresh YNB medium with different glucose levels. (B) Kinetic rearrangement of glucose transport upon cell shift from YPE to YNB with 1% glucose. (C) Fructose (Fru) transport in exponentially growing cells in YNB with low (0.1%) or high (1.0%) glucose. (D) Fructose transport 14 h after cell shift from YPE to YNB with 1% fructose. V, glucose (fructose) uptake rate in nmol min^{-1} mg^{-1} dry weight (DW). Hexose transport was measured as described in Materials and Methods with different concentrations of labeled sugars. (The different glucose or fructose concentrations used to measure transport [0.5, 5.0, and 50.0 mM] are indicated by bars of different colors.)

Hxs1 and Gcr1 and to which most of the known glucose transporters from various yeast species belong (exemplified on Fig. 1B with ScHxt1 and KlRag1). Hxt1, therefore, represents a plausible candidate for a functional hexose transporter in *H. polymorpha*.

Hxs1, as opposed to Hxt1, is nonfunctional as a hexose transporter in *S. cerevisiae*. To elucidate whether *H. polymorpha* hexose transporter homologues Hxs1 and Hxt1 are functional as hexose carriers, we overexpressed the corresponding genes under control of the *S. cerevisiae ADH1* promoter in the hexose transporterless mutant of *S. cerevisiae* (50), incapable of growing on hexoses (see Materials and Methods for details). We observed that only *HXT1* expression could functionally complement growth deficiency of this strain on glucose or fructose in the 5 to 100 mM range, while *HXS1* expression failed to do so (Fig. 2). Therefore, consistent with its sequence similarity, Hxt1 is a functional hexose transporter. On the contrary, as for the other yeast sugar sensors, Hxs1 is most probably a nontransporting protein.

HXS1 and HXT1 deficiency affects hexose transport. To elucidate the physiological importance of the two new transporter homologues, we assayed glucose uptake at different extracellular glucose concentrations in *H. polymorpha hxs1*Δ and *hxt1*Δ null mutants in comparison to the previously described *gcr1*Δ mutant (46) (Table 1; see Materials and Methods for details). The cells were preincubated for 2 h in YNB medium with either 1.0, 0.1, or 0.01% glucose before the assay of glucose uptake. We observed that in *hxs1*Δ and *hxt1*Δ cells adapted to 1% glucose, low-affinity glucose transport was strongly reduced, whereas high-affinity glucose transport was less affected. Glucose transport capacity was close to the wild-type rate in cells incubated with 0.1% or 0.01% glucose (Fig. 3A). Extended time course analysis revealed induction of low-affinity transport in *hxs1*Δ mutant cells growing on 1% glucose in batch culture, but after significant delay (Fig. 3B). Glucose transport capacities did not differ significantly between *hxs1*Δ and wild-type cells preincubated with glycerol or methanol (not shown). These results tentatively suggested that Hxt1 may function in *H. polymorpha* as a low-affinity glucose transporter and that its induction by glucose is mediated by the putative nontransporting sensor Hxs1. This is consistent with the sequence similarity data and functional analysis of the corresponding genes in the heterologous *S. cerevisiae* host (Fig. 2).

Interestingly, we observed that glucose-adapted *hxs1*Δ cells are completely deficient in fructose transport, which can be detected in the wild-type cells only after incubation with elevated glucose concentrations (Fig. 3C). Fructose transport was also dramatically impaired in *hxs1*Δ cells preincubated with fructose more strongly than glucose transport in glucose-

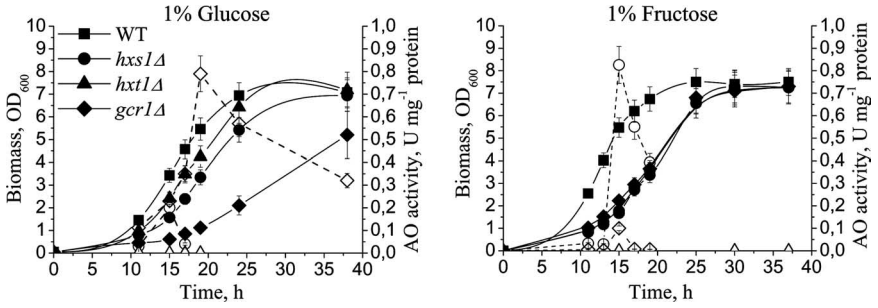

FIG. 4. *HXS1* and *HXT1* deficiencies have a moderate effect on growth on hexoses. Shown are the kinetics of growth and of AO activity in the wild-type (WT) strain and *hxs1*Δ, *hxt1*Δ, and *gcr1*Δ mutants on different carbon sources. The data represent the mean of two experiments. Cells of each strain were preincubated overnight in YPS (1% yeast extract, 2% peptone, 1% sucrose) medium and transferred to fresh media supplemented with 1% (wt/vol) of the indicated carbon sources. AO specific activity (open symbols) was measured in cell extracts prepared as described in Materials and Methods. Note the prolonged lag phase in *hxs1*Δ and *hxt1*Δ cells, as well as the pronounced defect in transient fructose repression of AO in *hxs1*Δ cells.

adapted cells (Fig. 3A and D). Therefore, Hxs1 is also important for proper regulation of fructose transport.

Both high- and low-affinity glucose and fructose uptake were significantly decreased in *gcr1*Δ cells preincubated with these sugars (Fig. 3A and D), which we included in the analysis for comparison. This observation is relevant to the question of whether catabolite repression is dependent on hexose transport, which is addressed in the next section.

Phenotypic analysis of *HXS1* and *HXT1* deletion strains. We investigated the role of Hxs1 and Hxt1 proteins in supporting optimal growth on different carbon sources. It was observed that the growth kinetics of the *hxs1*Δ and *hxt1*Δ strains in YNB medium with 1% glucose did not differ dramatically from that of the wild-type strain, except for a moderately prolonged lag phase (Fig. 4). The duration of lag phase was further extended in both mutants, whereas the cell doubling time in cells exponentially growing on 5% glucose was like that in the wild-type strain (not shown). Therefore, deficiency in *HXS1* or *HXT1* has a rather limited effect on glucose utilization. Hxt1 is most probably not the major transporter in *H. polymorpha*, required for short-term adaptation to glucose. We observed some decrease in cell doubling time in *hxs1*Δ and *hxt1*Δ cells grown with 1% fructose (Fig. 4). However, growth retardation was more evident in *hxs1*Δ cells incubated on solid media with these hexoses as carbon sources (see Fig. 8).

We also addressed the question of whether the partial decrease in hexose transport affects catabolite regulation in *hxs1*Δ and *hxt1*Δ mutants. Moderate levels of glucose-repressible peroxisomal AO protein and AO specific activity in glucose-grown *hxs1*Δ cells, but not *hxt1*Δ cells, could be detected only at the lag and early exponential growth phases and dropped sharply afterwards, suggesting short-term transient deficiency in glucose repression in the mutant (Fig. 4 and 5A). Consistent with the biochemical analysis, Q-PCR assays of expression of genes encoding AO (*MOX*) and glucose-repressible cytosolic maltase (*MAL1*) revealed a brief transient derepression in cells of *hxs1*Δ and *hxt1*Δ mutants shifted from ethanol to glucose (not shown). Apparently, such repression deficiency positively correlates with impaired glucose uptake, as it was much less pronounced in the *hxs1*Δ strain relative to the *gcr1*Δ strain used as a control (Fig. 3 and 4). Accordingly, *hxs1*Δ cells did not grow on methanol plates in the presence of 2-deoxyglucose, a nonmetabolizable glucose analogue that causes repression (46). Spontaneous 2-deoxyglucose-resistant mutants could be observed, originating with high frequency in the *hxs1*Δ background (Fig. 5B).

Remarkably, AO repression as measured by activity was

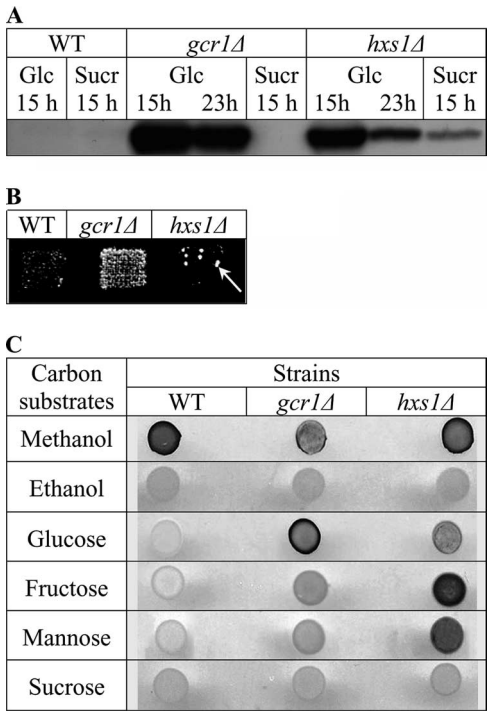

FIG. 5. Effect of the *hxs1*Δ mutation on catabolite repression triggered by different carbon sources. (A) Western blot detection of AO protein in cells incubated in glucose (Glc) or sucrose (Sucr) liquid media. Time points indicate time after shift from rich YPS medium supplemented with 1% (wt/vol) sucrose to YNB medium supplemented with 1% (wt/vol) of the corresponding carbon source. WT, wild type. (B) Growth of mutant cells on solid medium with 1% methanol and 150 mg/liter of 2-deoxyglucose (2-DG). Cells were incubated for 3 days. Spontaneous 2-DG-resistant mutants that appear in the *hxs1*Δ background are indicated with an arrow. (C) Visualization of AO activity in yeast colonies grown on different carbon sources (all 1% [wt/vol] or [vol/vol]). Cells were pregrown on YPS medium and replica plated on solid media with the selected carbon sources. Upon incubation for 24 h, AO activity was visualized by overlaying colonies with AO reaction mixture with permeabilizing agent (see Materials and Methods for details). The defect in fructose and mannose repression in *hxs1*Δ mutant is the most prominent finding.

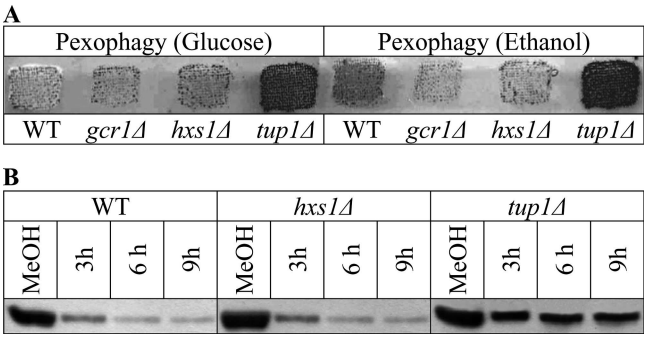

FIG. 6. Hxs1 is not required for signaling in autophagic peroxisome degradation (pexophagy) at onset of catabolite inactivation. (A) Visualization of AO activity in yeast colonies pregrown on methanol plates and replica plated onto the YNB solid media with glucose or ethanol (1% [wt/vol] and [vol/vol], respectively) to induce peroxisome degradation. Upon incubation for 12 h, AO activity was visualized by overlaying colonies with the AO reaction mixture with permeabilizing agent. WT, wild type. (B) Western blot detection of AO protein in cells shifted from methanol (MeOH) to fresh YNB glucose-containing medium to induce pexophagy. Time points indicate duration of adaptation to glucose after medium shift. Equal amounts of culture volumes were loaded for each strain at each time point.

more affected in the *hxs1*Δ strain than in the *gcr1*Δ strain in cells grown on fructose and mannose (Fig. 4 and 5C). On the other hand, the growth rates of *hxs1*Δ and *gcr1*Δ cells on fructose and also fructose transport were similar (Fig. 3 and 4). Therefore, although hexose uptake is important, it is not the sole factor determining transient repression proficiency for the corresponding sugar, at least for fructose.

AO repression by sucrose in *hxs1*Δ cells was only weakly affected: enzyme activity was below the detection level in sucrose-grown cells, and only traces of AO protein could be detected in the *hxs1*Δ strain (Fig. 5A and C). As could also be expected, ethanol remained a strong repressor of AO synthesis in both the *hxs1*Δ and *gcr1*Δ mutants (Fig. 5C).

We also addressed the question of whether *HXS1* is involved in (signaling for) another regulatory process triggered by glucose, namely, autophagic degradation of peroxisomes (pexophagy) that affects repressible gene products rapidly and directly at the protein level. A rapid decrease in peroxisomal AO protein level due to pexophagy can be observed upon adaptation of methanol-grown cells to glucose or ethanol (9). We observed that in methanol-preincubated *hxs1*Δ cells, AO activity and protein level decreased upon glucose adaptation with a rate similar to that of the wild-type strain (Fig. 6). The *H. polymorpha tup1* mutant deficient in pexophagy has been utilized as a positive control (25, 45). When methanol-preinduced *hxs1*Δ cells were shifted to fructose or ethanol, they also did not differ in the rates of AO degradation from the wild-type strain (not shown). Therefore, Hxs1, similarly to Gcr1 (Fig. 6) (46), is not essential for hexose signaling in catabolite inactivation by pexophagy.

Transcriptional regulation of the *H. polymorpha* hexose transporter genes by carbon sources. Our Northern blot and Q-PCR analyses revealed that Hxt1 expression in the wild-type strain is moderately transiently induced by elevated glucose concentrations, consistent with its predicted function as a low-affinity transporter (Fig. 7A and B). Hxt1 expression at any

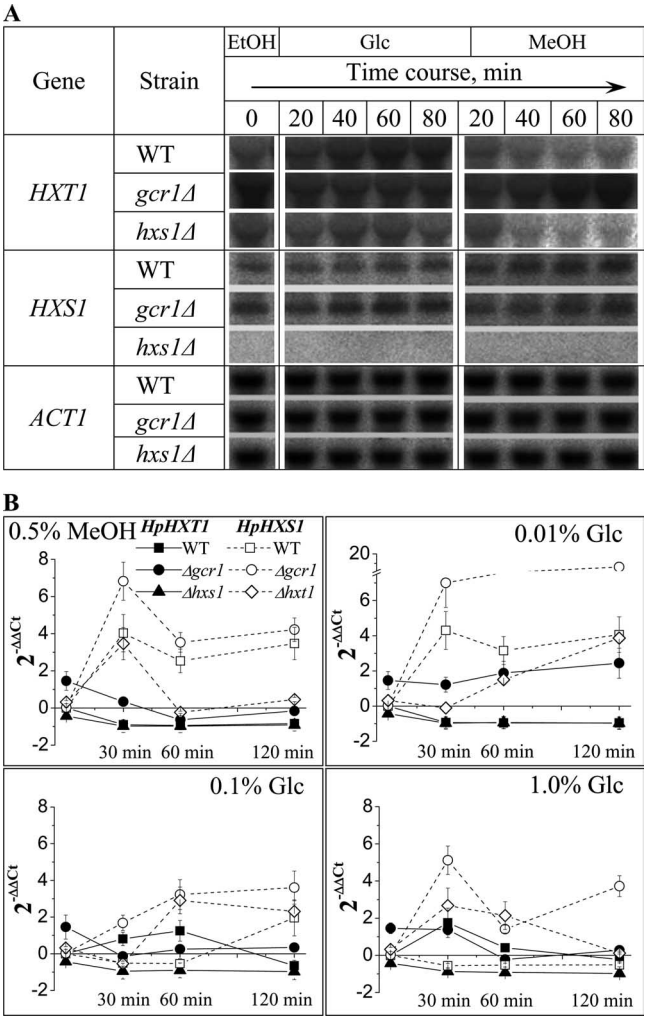

FIG. 7. Expression analysis of *HXT1* and *HXS1*. (A) Northern blot analysis. Cells of each strain were preincubated overnight in rich YPE medium with 1% ethanol (time point 0), washed, and shifted to YNB media with 1% (wt/vol) glucose or 1% (vol/vol) methanol. At indicated time points after the shift, cells were harvested for mRNA isolation and Northern blot analysis. Actin 1 gene (*ACT1*) expression was measured as a reference. Note that different amounts of mRNA were loaded for analysis of different genes: 1 μg of mRNA for *ACT1* analysis, 10 μg for *HXT1*, and 25 μg for *HXS1*, which exhibits the lowest expression level. The data suggest that *HXT1* is not induced efficiently by glucose in the *hxs1*Δ mutant and that *HXT1* is overexpressed in *gcr1*Δ cells in the absence of glucose. WT, wild type; EtOH, ethanol; MeOH, methanol. (B) Hxs1 is required for transient induction of *HXT1* by glucose and is derepressed in low glucose. The Q-PCR data represent the mean of two experiments performed as described in Materials and Methods. Cells grown to log phase in rich YPE medium with 1% (vol/vol) ethanol were diluted to an OD_{600} of 5.0 in YNB medium with the indicated carbon sources and incubated for 0.5, 1, and 2 h. The relative expression level indicated on the *y* axis ($2^{-\Delta\Delta C_T}$; calculated as described in reference 33) for each gene at each time point was normalized for its expression in YPE-grown wild-type cells ("0" on *x* and *y* axes).

glucose concentration was significantly diminished in the *hxs1*Δ mutant. Simultaneously, the *HXT1* gene was more highly expressed in low-glucose or methanol media in *gcr1*Δ cells. When the *hxs1*Δ mutation was introduced into *gcr1*Δ strain, this

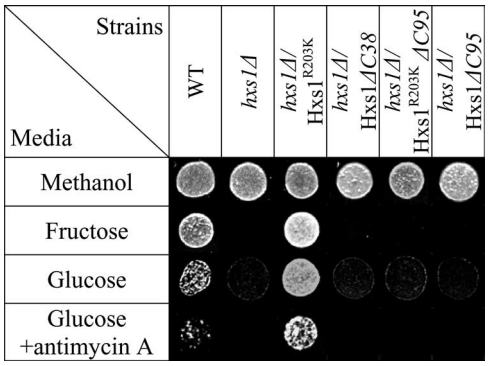

FIG. 8. Effect of the R203K mutation and of C-terminal truncation of Hxs1. Growth of strains expressing mutated forms of Hxs1 on different carbon sources is shown. Cells were pregrown on YPE plates and then spread with the same OD_{600} on YNB plates with the indicated carbon sources and incubated overnight; control methanol-grown cells were incubated for 2 days. The following substrate concentrations were used: methanol, 1% (vol/vol); fructose, 2% (wt/vol); glucose, 5% (wt/vol); and antimycin A, 6 mg/liter. The growth data suggest that R203K mutation converts the Hxs1 protein into constitutively signaling form that leads to better growth in high-glucose medium with the respiration inhibitor antimycin A, whereas C-terminal truncations in Hxs1 lead to complete loss of function. WT, wild type.

HXT1 overexpression was abolished (not shown). Therefore, Hxt1 induction by glucose clearly depends on intact Hxs1, which is consistent with our transport data (Fig. 3), whereas Gcr1 exerts a negative control over *HXT1* expression in the presence of low or no glucose.

The *HXS1* expression level in the cell was found to be very low irrespective of the carbon source, consistent with its function as a sensor protein (Fig. 7A). It is also differentially regulated by glucose: elevated glucose concentrations moderately repressed *HXS1* expression in the wild-type strain, whereas this repression was relieved in the *gcr1*Δ strains and, transiently, in *hxt1*Δ strains on high glucose or in a wild-type strain upon decreasing glucose concentrations (Fig. 7B).

One conserved amino acid substitution converts Hxs1 into a constitutively signaling form. It was previously demonstrated with ScSnf3, ScRgt2, and CaHgt4 that substitution of one of the conserved arginine residues in the hexose sensor's core sequence converts them into the constitutively signaling form in the absence of glucose (5, 31). We, therefore, constructed an Hxs1 allele with the corresponding (R203K) substitution (Fig. 1 and see Fig. S1 at http://www.cellbiol.lviv.ua/signal/articles/ec_hphxs1_supplement.pdf [see Materials and Methods for details]) and expressed it under the native *HXS1* promoter in the *hxs1* deletion mutant. We observed that $Hxs1^{R203K}$ efficiently complemented the growth defect on fructose plates in *hxs1*Δ cells (Fig. 8) and restored normal fructose repression of AO synthesis (not shown). However, the phenotype of $Hxs1^{R203K}$-expressing transformants differed from that of the wild-type cells in that they became more resistant to respiration inhibitor antimycin A in high-glucose medium (Fig. 8). We propose that this phenotype is caused by the constitutive overexpression of Hxt1 or other hexose transporters that support better glycolysis-dependent fermentative growth when respiration is inhibited. Therefore, mutated $Hxs1^{R203K}$ behaves in the same way as yeast glucose sensors that harbor an analogous mutation in this position (5, 31).

C-terminal extension of *HXS1* is required for its sensing function. Finally, we constructed truncated forms of Hxs1 lacking portions (38 or 95 amino acid residues) of its C terminus (Fig. 1 and see Fig. S1 at http://www.cellbiol.lviv.ua/signal/articles/ec_hphxs1_supplement.pdf for reference) and expressed them from the native gene promoter in the *hxs1*Δ mutant. We observed that deletion of at least 38 extreme C-terminal amino acid residues renders Hxs1 nonfunctional: the truncated protein did not overcome the *hxs1*Δ growth deficiency on fructose (Fig. 8) and defect in AO repression on this substrate (not shown). This 38-amino-acid-residue deletion does not comprise but is adjacent to the Hxs1 fragment similar in its hydrophobicity profile to the corresponding fragment containing the "glucose sensor domain" of other yeast sensors (Fig. 1 and see Fig. S1 at http://www.cellbiol.lviv.ua/signal/articles/ec_hphxs1_supplement.pdf). Moreover, deletion of the 95 C-terminal amino acid residues in an allele harboring the constitutively signaling $Hxs1^{R203K}$ form also produced a nonfunctional protein: transformants expressing $Hxs1^{R203K}$Δ95C did not differ in their phenotype from the recipient *hxs1*Δ mutant (Fig. 8). Hence, these observations demonstrate that, similarly to glucose sensors from other yeasts, the hydrophilic C-terminal region of Hxs1 is strictly essential for its function (5, 31).

DISCUSSION

In this report, we describe a hexose transporter homologue from the methylotrophic yeast *H. polymorpha*, designated *HXS1* (*h*exose *s*ensor), that apparently fulfils a hexose-sensing function in this yeast. Hxs1 belongs to a distinctive subclade of hexose transporters which contains all known to date orthologous yeast transporter-like glucose sensors, namely *S. cerevisiae* Snf3 and Rgt2 (32), *K. lactis* Rag4 (4), and *C. albicans* Hgt4 (5) (Fig. 1). Similarly to other Crabtree-negative yeasts, *K. lactis* and *C. albicans*, the *H. polymorpha* genome apparently harbors only a single such sensor. The sensor proteins appear to be phylogenetically related to yeast and fungal high-affinity hexose symporters, although not as closely as our previously identified *H. polymorpha* hexose transporter homologue Gcr1 (46) (Fig. 1B).

Besides the apparent primary sequence similarity, several experimental observations support a hexose sensor function for Hxs1. (i) The corresponding gene is expressed at a moderately low level and is up-regulated upon glucose depletion. (ii) Hxs1, like *S. cerevisiae* Snf3 and Rgt2 and *K. lactis* Rag4, which are intrinsically unable to transport glucose (4, 32), is nonfunctional as hexose permease in a heterologous *S. cerevisiae* system. (iii) Glucose induction of *H. polymorpha* functional transporter Hxt1 is Hxs1 dependent. (iv) Truncated Hxs1 protein lacking portions of its C-terminal sequence is unable to functionally complement the *hxs1*Δ deletion mutant. (v) One conserved amino acid substitution (R203K) converts Hxs1 into a constitutively signaling form that leads to elevated resistance to the respiration inhibitor antimycin A on high-glucose medium, probably due to the overexpression of hexose transporters that support fermentative growth. Preliminary unpublished results also suggest that, besides Hxt1, which itself is capable of mediating fructose

transport in *S. cerevisiae* (Fig. 2), Hxs1 is required for induction/derepression of at least one other putative transporter in *H. polymorpha*, highly homologous (52% identity and 68% similarity) to the recently characterized specific yeast fructose transporter *K. lactis* Frt1 (8).

It can be concluded that *H. polymorpha*, similarly to other yeasts, possesses a glucose-sensing system for transcriptional regulation of functional hexose transporter(s) in response to glucose availability facilitated by a nontransporting receptor. Whether the downstream molecular components of this pathway in *H. polymorpha* are the same as those described in *S. cerevisiae*, *K. lactis*, and *C. albicans* (i.e., functional homologues of Yck1 casein kinase I, Mth1/Std1, and the Rgt1 transcriptional factor) (29, 37, 42) remains to be elucidated. The corresponding genes that potentially encode all of these orthologues are present in the *H. polymorpha* genome (our unpublished observation). Also, our analysis of the predicted *HXT1* promoter region revealed at least six putative consensus binding sites of the ScRgt1 repressor (18), suggesting that the sensor-dependent pathway may indeed have the same conserved components in different ascomycetous yeasts.

Remarkably, deletion of the *HXS1* gene does not lead to a significant decrease in growth rate on hexoses in liquid media except for a prolonged lag phase (Fig. 4). Nevertheless, the growth deficiency in the *hxs1*Δ mutant is evident on solid media with hexoses after short-term incubation (Fig. 8). We also observed that the transient defect in fructose repression of the *hxs1*Δ mutant is prominent, whereas the effect of the mutation on glucose repression is minimal. The plausible explanation for such "fructose-specific" phenotype of the *hxs1*Δ mutant, reminiscent of the phenotype described for the *C. albicans hgt4*Δ strain (5), may be that proficiency of hexose-triggered catabolite repression in *H. polymorpha* depends on the transport capacity for the corresponding sugar, rather than hexose-sensing function. Indeed, the defect in both components (low and high affinity) of glucose transport and the defect in glucose repression are both much more pronounced in the *gcr1*Δ mutant than in the *hxs1*Δ mutant (Fig. 3 to 5). Also, the defect in glucose repression was enhanced in the double *gcr1*Δ *hxs1*Δ mutant (unpublished observation). Consistent with the proposed hypothesis, high- and low-affinity fructose transport in *hxs1*Δ cells is severely impaired (approximately 10 times decreased relative to the wild-type strain), whereas only low-affinity glucose transport is partially affected in this mutant (Fig. 3A and D). It has been previously established that hexose phosphorylation is required for the repression pathway in *H. polymorpha* (20). Therefore, the intracellular level of the effector hexose as influenced by its uptake rate may determine the strength of the downstream repression signal (e.g., the rate of hexose phosphorylation).

Nevertheless, a set of data indicates that the hexose repression mechanism in *H. polymorpha* is more complex and may also involve some form of sensing of the effector substrate. For instance, fructose transport capacities do not differ dramatically between *hxs1*Δ and *gcr1*Δ mutants, whereas transient repression deficiency does, being more pronounced in the former (Fig. 3 and 4). It is plausible that differential involvement of hexose-phosphorylating enzymes hexo- and glucokinase, in response to different hexoses and/or their Hxs1-dependent regulation (5), may underlie the observed effects. With the available data, we propose that hexose transport is important but is not the sole cause affecting repression signaling in *H. polymorpha*.

In this report, we also describe the first functional hexose transporter identified in *H. polymorpha*, Hxt1 (*h*e*x*ose *t*ransporter). Our results favor its physiological function as a low-affinity hexose transporter. (i) The Hxt1 protein is highly similar to functional low-affinity transporters from other yeasts. (ii) Its expression in *H. polymorpha* is induced by high glucose concentrations. (iii) Upon overexpression, it supports growth of the *S. cerevisiae* hexose transporter-deficient strain on glucose and fructose in the mM range. (iv) A mutant deleted for the *HXT1* gene exhibits a decrease in low- but not high-affinity glucose uptake. Our observations are in agreement with previous reports that suggested the presence of a low-affinity kinetic component of hexose transport in *H. polymorpha* (17). From the results obtained with the *hxt1*Δ mutant, we can conclude that Hxt1 is rather redundant for growth on glucose and fructose (Fig. 4). Nevertheless, the *hxt1*Δ deletion mutant, similarly to the *hxs1*Δ mutant, exhibited weak transient derepression of glucose-repressible genes (*AOX1* and *MAL1*) upon shift of ethanol-grown cells to high-glucose medium (unpublished results obtained with Q-PCR), suggesting that this transporter may be important for supporting the hexose transport rate required to establish physiological transcriptional regulation upon short-term glucose adaptation.

Quite interestingly, *HXT1* is overexpressed in the *gcr1*Δ mutant in ethanol and methanol media (Fig. 7A). Such a physiological effect in the absence of glucose is rather unexpected if Gcr1 is a regular hexose transporter. We previously suggested that Gcr1, which is considerably similar in sequence to yeast glucose sensors but is "tail-less," may fulfill a glucose-sensing function for catabolite repression in *H. polymorpha* (46). Recently, however, several fungal and yeast high-affinity glucose symporters, to which Gcr1 is more closely related, as judged by sequence similarity (Fig. 1), have been described in the literature: i.e., *Aspergillus niger* MSTA (49). The intriguing question of whether Gcr1 may have a dual function as a transporting receptor will be addressed in a separate study.

It has to be mentioned that the hexose transport system in *H. polymorpha* is apparently quite complex. Besides the genes studied in this paper, at least six other genes potentially encoding hexose transporters, as well as two genes potentially encoding specific fructose transporters, were identified in the *H. polymorpha* genome sequences (unpublished observations). To evaluate their role in hexose transport and/or sensing, these genes have to be functionally characterized.

In addition to transcriptional regulation, glucose also triggers catabolite inactivation of the repressible peroxisomal enzymes that involves degradation of peroxisomes in vacuoles (pexophagy) (9). We demonstrated that Hxs1, similarly to Gcr1 (46), is not essential for this regulatory pathway. These data strengthen the notion that molecular triggers of pexophagy may primarily depend on glycolytic flux and, consequently, cell energy status (2), rather than membrane-bound sensing of the effector hexose (3) or its transport. However, GPCR-mediated sensing for pexophagy cannot be excluded (30).

Methylotrophic yeasts, especially *H. polymorpha* and *P. pastoris*, are popular objects in basic science and biotechnology (9, 14, 24, 38). Further understanding of the different glucose-

sensing mechanisms in these and other nonconventional yeasts would shed light on the versatility of these systems and undoubtedly will be useful for a variety of biotechnological applications.

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