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The expression in *Saccharomyces cerevisiae* of a glucose/xylose symporter from *Candida intermedia* is affected by the presence of a glucose/xylose facilitator

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Two glucose/xylose transporter genes from *Candida intermedia* were recently cloned and characterized: *GXF1*, which encodes a glucose/xylose facilitator; and *GXS1*, which encodes a glucose/xylose proton symporter. Here we report the functional expression of these transporters in *Saccharomyces cerevisiae*. While Gxf1p seems to be fully functional in *S. cerevisiae*, the symporter Gxs1p exhibits very low glucose/xylose transport activity, which could not be ascribed to insufficient production of the protein or incorrect subcellular localization. In addition, co-expression of glucose/xylose facilitators with Gxs1p strongly reduced *GXS1* mRNA levels, and consequently symport activity, in glucose-grown, but not in ethanol-grown, cells. The observed decrease in *GXS1* transcript levels seems to be related to an enhanced glucose influx mediated by glucose facilitator protein(s), and not to a specific interaction between Gxs1p and other transporters. We found *GXS1* mRNA levels to be severely reduced as a result of glucose addition, and we show that this effect takes place at the level of *GXS1* mRNA stability. Our results suggest that a decrease in mRNAs encoding high-affinity/active sugar transport systems may be a widespread and conserved mechanism in yeasts, limiting expression of these proteins whenever their activity is dispensable.

INTRODUCTION

The production of fuel ethanol from hemicellulose hydrolysates presents clear advantages from both an environmental and an economic point of view (Hahn-Hägerdal *et al.*, 2006; Jeffries, 2006; van Maris *et al.*, 2006). *Saccharomyces cerevisiae* has been considered to be the organism with the highest potential for the production of ethanol from biomass hydrolysates, mainly because of its efficiency and robustness. However, *S. cerevisiae* is naturally unable to use pentose sugars as carbon and energy sources, which constitutes a major drawback, since hemicellulose hydrolysates contain high amounts of xylose and arabinose. Therefore, considerable research has focused on the metabolic engineering of *S. cerevisiae* to co-ferment the hexoses and pentoses derived from lignocellulosic feedstocks. Recombinant xylose-fermenting *S. cerevisiae* strains have been obtained by combining the expression of heterologous enzymes, either from the yeast *Pichia stipitis* (xylose reductase and xylitol dehydrogenase) or from the filamentous fungus *Piromyces* sp. (xylose isomerase), with the modulation of native enzymes that lead to positive effects on fermentation efficiency (Jeffries, 2006; van Maris *et al.*, 2006; Hahn-Hägerdal *et al.*, 2007).

In all the recombinant yeast strains currently available, xylose uptake occurs solely via the hexose facilitators (Hxt proteins and Gal2) that also accept xylose as a substrate, albeit with very low affinity (K_m >100 mM; Hamacher *et al.*, 2002). For this reason, xylose uptake is slow and dependent on the concentration of glucose present in the environment. Notably, an engineered *S. cerevisiae* strain selected for efficient xylose fermentation exhibits significantly improved xylose transport kinetics (Kuyper *et al.*, 2005). Heterologous expression of a high-affinity xylose transporter is therefore expected to represent an asset for xylose-fermenting *S. cerevisiae* strains in which other limiting factors have already been optimized (Gárdonyi *et al.*, 2003a; Karhumaa *et al.*, 2006).

An attempt to isolate xylose transporters from *P. stipitis* by functional complementation yielded the low-affinity Sut1–3 transporters (Weierstall *et al.*, 1999), but the isolation of a high-affinity xylose transporter from the same yeast remains elusive, despite the recent sequencing of its genome (Jeffries *et al.*, 2007). Recently, we reported the cloning and characterization of two glucose/xylose transporter genes from *Candida intermedia*, designated *GXF1*

Abbreviation: UTR, untranslated region.

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and *GXS1* (Leandro *et al.*, 2006). *GXF1* encodes a glucose/xylose facilitator, whereas *GXS1* encodes a glucose/xylose proton symporter, with K_m values of 50 and ~0.4 mM, respectively. While Gxf1 functions very well in the heterologous context, *S. cerevisiae* expressing the symporter protein displays very weak transport activity. The different performances raise questions regarding the identification of factors hampering functional expression of the symporter, but not of the facilitator, in the host environment.

Deficient targeting of heterologous transporters to the plasma membrane has often been observed in *S. cerevisiae* (Wieczorke *et al.*, 2003). Moreover, an apparent relationship between low- and high-affinity sugar transport systems has been detected in different yeasts (Spencer-Martins & van Uden, 1985; Cason *et al.*, 1986; Lucas & van Uden, 1986; Verma *et al.*, 1987; Ramos *et al.*, 1988; Walsh *et al.*, 1994), and these studies suggest that expression of transporter proteins is stringently regulated. In *S. cerevisiae*, a complex picture has emerged after the discovery of a surprisingly extensive set of *HXT* genes involved in hexose transport. Regulatory mechanisms governing the activity of the most important members of this family include both transcriptional regulation and specific targeting of transporters for degradation, dictated mainly by the sugar concentration in the growth medium (Gancedo, 1998; Rolland *et al.*, 2002; Kresnowati *et al.*, 2006).

We now report on various aspects of the expression of the two aforementioned *C. intermedia* transporters in *S. cerevisiae* (transcription and translation efficiencies, subcellular localization and mRNA stability), with special attention given to the possible causes of the poor performance of the glucose/xylose proton symporter, and in particular addressing the co-expression of both transporters.

METHODS

Yeast strains and growth conditions. The strains used or generated in this work are listed in Table 1. Yeast strains were routinely grown in YNB (yeast nitrogen base without amino acids; Difco) medium containing the indicated carbon source and the required supplements at 25 °C and 150 r.p.m. unless otherwise stated. Growth of the cultures was followed by monitoring OD_{640} with an Ultrospec 3100 pro spectrophotometer (GE Healthcare) (OD_{640}=1 corresponds to ~0.3 mg dry weight per ml). Sugar concentrations are given as percentages (w/v). Strains TMB 3201 and TMB 3001 were provided by Professor Bärbel Hahn-Hägerdal, University of Lund, Sweden, and strain C5 was provided by Professor Uwe Sauer, ETHZ, Switzerland.

Symport assays. Symport assays were performed as described previously (Fonseca *et al.*, 2007). Cultures were harvested at an OD_{640} of ~0.7. Symport activity was assessed by the proton influx elicited by the addition of either glucose or xylose to an aqueous cell suspension adjusted to pH 5.

Isolation of yeast plasma membranes and Tricine SDS-PAGE. *C. intermedia* PYCC 4715 cells cultivated in Verduyn medium (Verduyn *et al.*, 1992) containing 0.5 % xylose, 2 % glucose or 4 % xylose, and *S. cerevisiae* strains MJY1, MJY2, MJY5 and MJY6 cultivated in Verduyn medium with 2 % glucose, were harvested during the exponential growth phase (OD_{640} 1–2). Plasma and mitochondrial membranes were isolated as described previously (Leandro *et al.*, 2006). Protein concentrations were determined using the BCA Protein Assay kit (Pierce). Aliquots of plasma membrane preparations containing 20 μg total protein were separated by Tricine SDS-PAGE (Schägger, 1994) in a 7.5 % polyacrylamide/0.23 % bisacrylamide gel.

Plasmid and strain construction. A DNA fragment consisting of the *GFP* ORF with short flanking regions (45 bp each) homologous to the 3′ end of the *GXS1* ORF and to the beginning of the *PGK1* terminator sequence was amplified by PCR using plasmid pT5II-GFP-GAP (Rodrigues de Sousa *et al.*, 2004) as the template and primers GXS1-GFP_For (5′-ATGGACTCCAAAACTGAAGCTATTATGT-CTGAAGAAGCTTCTGTTAGTAAAGGAGAAGAACTTTTC-3′) and

Table 1. Yeast strains used in this study

Strain	Genotype	Reference
C. intermedia PYCC 4715		Gárdonyi *et al.* (2003b)
S. cerevisiae TMB 3201	*MATa Δhxt1-17 Δgal2 Δstl1 Δagt1 Δmph2 Δmph3 leu2-3,112 ura3-52 trp1-289 his3-Δ1::YIpXR/XDH/XK MAL2-8^c SUC2*	Hamacher *et al.* (2002)
S. cerevisiae TMB 3001	*MATa his3- Δ1::YipXR/XDH/XK MAL2-8^c SUC2*	Eliasson *et al.* (2000)
S. cerevisiae C5	Isolated from evolved TMB 3001 population	Sonderegger & Sauer (2003)
MJY1	TMB 3201 + pGXF1	Leandro *et al.* (2006)
MJY2	TMB 3201 + pHXT7-GXS1	Leandro *et al.* (2006)
MJY3	TMB 3201 + pPGK-GXS1	Leandro *et al.* (2006)
MJY5	TMB 3201 + pPGK-GXS1 + pGXF1	Leandro *et al.* (2006)
MJY6	TMB 3201 + pHXT7-GXS1–GFP	This study
MJY7	TMB 3001 + pHXT7-GXS1	This study
MJY8	TMB 3001 + pHXT7-GXS1–GFP	This study
MJY9	C5 + pHXT7-GXS1	This study
MJY12	TMB 3201 + p(181)HXT7-GXS1	This study
MJY13	TMB 3201 + p(204)HXT7-GXS1-i	This study
MJY15	TMB 3201 + p(181)HXT7-GXS1 + pGXF1	This study
MJY16	TMB 3201 + p(204)HXT7-GXS1-i + pGXF1	This study

GFP-TER_Rev (5′-GAAAAGAAAAAAATTGATCTATCGATTTCAA-TTCAATTCAATTTACTATTTGTATAGTTCATCC-3′). The *hxt*-null *S. cerevisiae* TMB 3201 strain was simultaneously transformed with this PCR product and with plasmid pHXT7-GXS1 (Leandro *et al.*, 2006), which had been linearized previously with *Xba*I. Ura$^+$ transformants were first recovered in YNB maltose medium and subsequently plated onto medium containing glucose as the sole carbon and energy source. One of the transformants obtained, strain MJY6, was selected for further studies.

Plasmid pHXT7-GXS1–GFP was recovered from strain MJY6 and was used to transform ura$^-$ mutants (obtained through positive selection on medium containing 5-fluoroorotic acid) of *S. cerevisiae* strain TMB 3001 (Eliasson *et al.*, 2000), resulting in strain MJY8.

The ura$^-$ mutants of *S. cerevisiae* strains TMB 3001 and C5 (Sonderegger & Sauer, 2003) were transformed with the high-copy-number plasmid pHXT7-GXS1 (Leandro *et al.*, 2006), producing strains MJY7 and MJY9, respectively.

Plasmid pHXT7-GXS1 (Leandro *et al.*, 2006) was digested with *Pst*I and *Sac*I to release the HXT7(promoter)-GXS1(ORF)-PGK(terminator) insert that was then subcloned into either 2μ plasmid YEplac181 or integrative plasmid YIplac204 (Gietz & Sugino, 1988), between the *Pst*I and *Sac*I sites, resulting in plasmids p(181)HXT7-GXS1 and p(204)HXT7-GXS1-i, respectively. Strain MJY12 was obtained by transformation of *S. cerevisiae* TMB 3201 with plasmid p(181)HXT7-GXS1. Strain MJY15 was obtained by transformation of strain MJY12 (TMB 3201 + p(181)HXT7-GXS1) with plasmid pGXF1 (Leandro *et al.*, 2006). Strain MJY13 was obtained by transformation of *S. cerevisiae* TMB 3201 with integrative plasmid p(204)HXT7-GXS1-i digested with *Eco*RV. Strain MJY16 was obtained by transformation of strain MJY13 (TMB 3201 + p(204)HXT7-GXS1-i) with plasmid pGXF1 (Leandro *et al.*, 2006).

Northern blotting. Cultures were harvested at OD_{640} ~0.7 and frozen in liquid nitrogen prior to RNA isolation. Total RNA isolation and gel electrophoresis were performed as described elsewhere (Griffioen *et al.*, 1996). The gels were blotted onto Hybond-N nylon membranes (Amersham Biosciences). A 163 bp fragment of the *GXF1* gene and a 300 bp fragment of the *GXS1* gene were used as gene-specific probes, as described previously (Leandro *et al.*, 2006). A 733 bp fragment of the *PDA1* (pyruvate dehydrogenase) gene, amplified from genomic DNA of *S. cerevisiae* TMB 3201 with primers PDAP1 (5′-ATGGCTTGTGACGCCTTGTA-3′) and PDAP2 (5′-GACCAGCAATTGGATCGTTC-3′), was used as a *PDA1*-specific probe. A 192 bp fragment of the *ACT1* (actin) gene, amplified from genomic DNA of *S. cerevisiae* TMB 3201 with primers ActFor (5′-AGTGAACGTGGTTACTCTTTC-3′) and ActRev (5′-GGCTCTG-AATCTTTCGTTAC-3′), was used as an *ACT1*-specific probe. Probes were labelled with either [α-^{32}P]ATP or [α-^{32}P]CTP (Amersham Biosciences) using the Prime-a-Gene Labelling system (Promega). Hybridization and washing conditions were as described elsewhere (Griffioen *et al.*, 1996).

Inhibition of transcription with 1,10-phenanthroline. For inhibition of transcription the method described by Parker *et al.* (1991) was used. Yeast was cultivated at 25 °C in 1 l YNB medium containing 1 % (w/v) ethanol up to OD_{640} ~0.8. A 200 ml volume was kept on ice to prepare cell suspensions for the determination of symport activity. The remaining 800 ml was divided into two aliquots, each subsequently concentrated 10× in the same medium. Either 1,10-phenanthroline alone, to a final concentration of 100 μg ml^{-1}, or 1,10-phenanthroline plus glucose (2 %, final concentration) was added to the aliquots. Samples (4 ml) were collected immediately following addition of inhibitor (time 0) and after incubation for 5, 10, 20, 40 and 60 min at 25 °C. The cell pellets were rapidly frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated from the frozen cells using TRIzol reagent (Invitrogen).

Microscopy. Live GFP-transformed cells were spotted onto microscope slides, and epifluorescence images were acquired using an Olympus BX50 microscope equipped with a U-ULH 100 W mercury high-pressure bulb and a U-MWIB2 filter set for GFP (Olympus). Images were obtained with a digital camera (Olympus C3030-ZOOM).

Miscellaneous. Molecular biology techniques were performed using standard protocols (Sambrook *et al.*, 1989). Yeast transformations were performed using the lithium acetate method (Gietz & Woods, 2002). Restriction enzymes and ligase were purchased from Roche. *Escherichia coli* strain MOSBlue (Amersham Biosciences) was used as the host for DNA manipulations. Primers were obtained from MWG Biotech.

RESULTS

GXS1 transcript levels versus symport activity in *S. cerevisiae*

Although the *GXS1*-encoded glucose/xylose symporter from *C. intermedia* proved to be functional in *S. cerevisiae* TMB 3201 (Leandro *et al.*, 2006), a strain devoid of any other glucose/xylose transporters (Hamacher *et al.*, 2002), its activity was very low both when expressed from a low-copy-number or from a high-copy-number vector. This was consistently observed for the several independent transformants examined. Expression was driven by a strong constitutive promoter, either a truncated version of the *HXT7* promoter (Hamacher *et al.*, 2002) or the *PGK1* promoter (Kingsman *et al.*, 1990), which should result in high constitutive transcription levels. Nevertheless, to evaluate whether the weak Gxs1 transporter activity in the *S. cerevisiae* transformants was due to low mRNA levels, we determined both symport activity and mRNA levels in the original *C. intermedia* PYCC 4715 strain from which the gene was isolated and in *S. cerevisiae* MJY2 (Leandro *et al.*, 2006) expressing Gxs1p from a multicopy vector. Although mRNA levels are similar in the *S. cerevisiae* transformant carrying the multicopy plasmid and in *C. intermedia* (Fig. 1), xylose symport activity was considerably higher in the latter (Fig. 2). In contrast, it should be

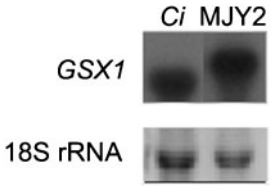

Fig. 1. *GXS1* transcript levels. Northern blot analysis of *GXS1* expression in *C. intermedia* PYCC 4715 (*Ci*) cultivated in 0.5 % xylose, and in *S. cerevisiae* MJY2 cultivated in 2 % glucose. Northern blots were probed with a *GXS1*-specific probe. Ethidium bromide staining of the RNA gel is shown as a loading control.

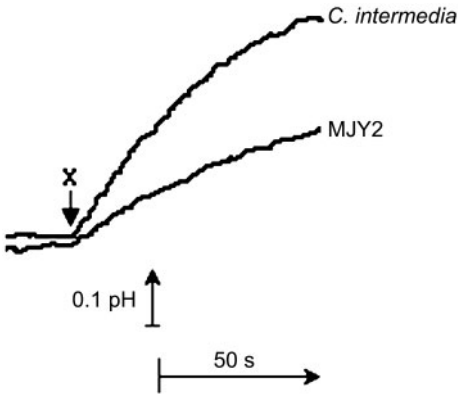

Fig. 2. Xylose symport activity. The effect on extracellular pH is shown of xylose added to a final concentration of 6.7 mM to aqueous cell suspensions of *C. intermedia* PYCC 4715 cultivated in 0.5 % xylose and *S. cerevisiae* MJY2 cultivated in 2 % glucose. X, time of sugar addition.

noted that expression of Gxf1p, the glucose/xylose facilitator also isolated from *C. intermedia*, resulted in similar mRNA levels in both yeasts but much higher glucose/xylose transport rates in *S. cerevisiae* (Leandro *et al.*, 2006). We therefore concluded that the poor symport activity was not due to lack of *GXS1* mRNA in *S. cerevisiae* transformants under the conditions examined. This finding led us to examine the role of post-transcriptional events in the expression of Gxs1p.

In *C. intermedia*, just like in the well-known human pathogen *Candida albicans*, a deviation from the standard genetic code occurs, which consists of decoding the leucine CUG codon as serine (Sugita & Nakase, 1999). Both sequences of the *GXS1* and *GXF1* genes were screened for the utilization of this codon, but neither gene encoded leucine as CUG. Since the two *C. intermedia* genes are very similar in codon usage despite the difference in transport activities when expressed in *S. cerevisiae*, codon bias was also excluded as a possible source of poor Gxs1 activity.

Protein levels of *C. intermedia* transporters in *S. cerevisiae*

It has been often observed that plasma membrane proteins expressed in an heterologous context may be deficiently targeted or present serious problems for the secretion pathway of the host cell, in particular when overexpressed (Wieczorke *et al.*, 2003; Flegelova *et al.*, 2006). To examine this question, we constructed a Gxs1–GFP fusion for use as a reporter for the subcellular localization of heterologously expressed Gxs1p. In most living cells of strain MJY6, which expresses the fusion protein from the same multicopy expression system present in strain MJY2 (expressing *GXS1* only; Leandro *et al.*, 2006), fluorescence could clearly be observed at the periphery of the cell, as would be expected for a protein targeted to the plasma membrane (Fig. 3).

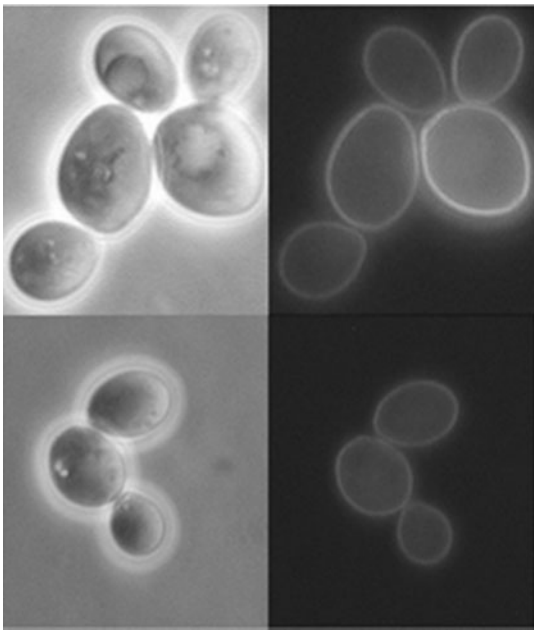

Fig. 3. Subcellular localization of Gxs1–GFP. Phase-contrast (left panel) and epifluorescence (right panel) images are shown of *S. cerevisiae* strain MJY6 cells (*hxt*-null strain TMB 3201 transformed with multicopy plasmid pHXT7-GXS1–GFP) cultivated on 2 % glucose.

Moreover, strain MJY6 grows on glucose, thereby confirming that the Gxs1–GFP fusion protein is a functional glucose transporter located in the plasma membrane. To gain insight into the relative abundance of heterologous Gxs1p and the Gxs1–GFP fusion in the plasma membrane of *S. cerevisiae*, plasma membranes were isolated from strains MJY2 and MJY6 and run on SDS-PAGE optimized for the separation of membrane proteins. A comparative analysis of the band patterns showed that the clearly noticeable Gxs1p band present in plasma membranes isolated from *C. intermedia* cells grown in 0.5 % xylose (inducing conditions; Gárdonyi *et al.*, 2003b; Leandro *et al.*, 2006) also appears in the plasma membrane fraction of strain MJY2 as a highly prominent band (Fig. 4). In the membrane of strain MJY6 a strong band is visible corresponding to a molecular mass of approximately 67 kDa, which is the expected molecular mass of the fusion protein Gxs1–GFP (an increase of ~27 kDa due to the GFP moiety). This demonstrates that both native Gxs1p and the Gxs1–GFP fusion protein are abundantly produced and correctly directed to the plasma membrane. Indeed, Gxs1p appears to be the second most abundant protein in the plasma membrane, following the plasma membrane ATPase which corresponds to the very prominent band at ~95 kDa (Fig. 4).

We have also expressed Gxs1p and the Gxs1–GFP fusion in a strain carrying a genomic mutation that was selected for improving functional expression of the mammalian

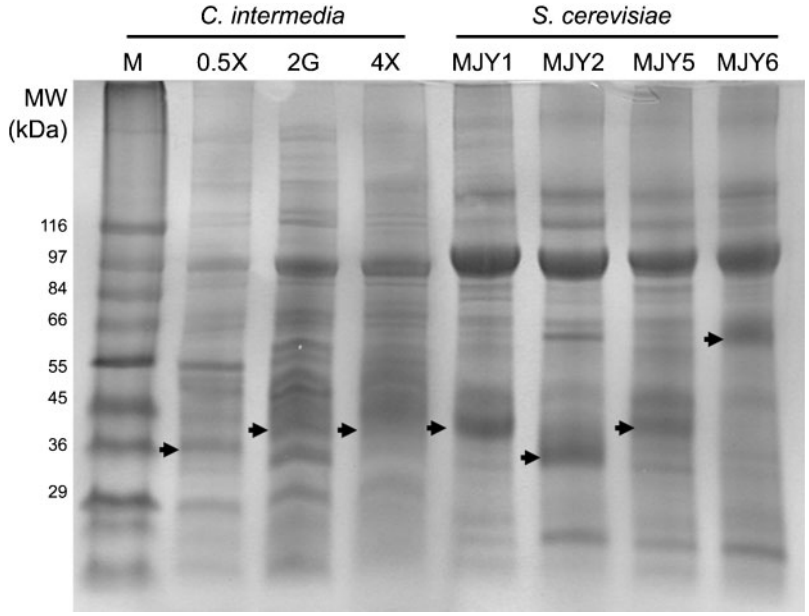

Fig. 4. Abundance of glucose/xylose transporters. Tricine SDS-PAGE is shown of plasma membrane proteins isolated from *C. intermedia* PYCC 4715 cells cultivated on 0.5 % (0.5X) or 4 % (4X) xylose or 2 % glucose (2G), and from *S. cerevisiae* MJY1, MJY2, MJY5 and MJY6 cells cultivated in 2 % glucose. The gel was stained with Coomassie Blue. M, molecular mass marker (Sigma-Aldrich, Wide Range). The arrows indicate the positions of bands corresponding to Gxs1p (lanes 0.5X and MJY2), Gxf1p (lanes 2G, 4X, MJY1 and MJY5), and Gxs1p–GFP (lane MJY6).

GLUT1 transporter in *S. cerevisiae* (strain EBY.S7; Wieczorke *et al.*, 2003). The transformants expressing Gxs1–GFP exhibited a clear retention of fluorescence in intracellular structures, suggesting that this mutation affects trafficking of Gxs1–GFP to the plasma membrane (results not shown).

We concluded, therefore, that neither an incorrect subcellular localization nor insufficient production of the protein accounts for the observed deficit in Gxs1p-mediated glucose/xylose symport activity in *S. cerevisiae* TMB 3201.

Gxs1 expression in the presence of glucose/xylose facilitators

In the course of our attempts to improve functional expression of Gxs1p in *S. cerevisiae*, different strain backgrounds were tested, among which were two strains that grow relatively well on xylose, TMB 3001 (Eliasson *et al.*, 2000) and C5 (Sonderegger & Sauer, 2003). Both strains differ from the host used to construct MJY2 and MJY6 in that they carry all native *S. cerevisiae* hexose transporters (Hxt proteins). Strains TMB 3001 and C5 were transformed with plasmid pHXT7-GXS1, yielding strains MJY7 and MJY9. Surprisingly, we failed to detect symport activity in glucose-grown cells of both strains. We reasoned that the absence of symport activity might be related to the presence in the host strains of the hexose transporters operating through facilitated diffusion. This could be the result of a specific interaction between Gxs1p and one or more of the Hxt proteins, or could be the consequence of an increased glucose uptake in these strains. In the latter case this effect would be expected to be independent of the particular functional transporter present. To investigate this possibility, we compared symport activity in two *hxt*-null strains (Leandro *et al.*, 2006): MJY12, carrying only the *GXS1* symporter gene; and MJY15, carrying both the *GXS1* (symporter) and the *GXF1* (facilitator) *C. intermedia* genes. In MJY15, unlike in MJY12, we failed to detect symport activity (Fig. 6a). This is in line with the view that the presence of an efficient facilitated glucose transport system may cause a decrease in Gxs1 activity. We subsequently analysed, by Northern blotting, whether this decrease took place at the mRNA level. In these experiments (Fig. 5) we examined both *GXF1*

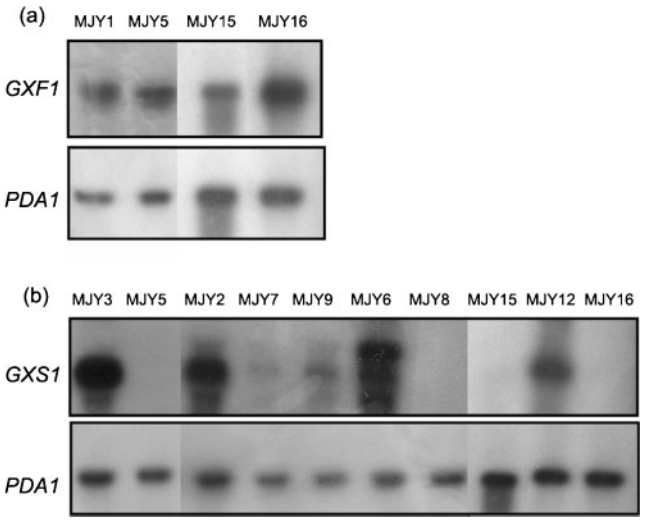

Fig. 5. Co-expression of Gxs1 and facilitators. Northern blot analysis of *GXF1* (a) and *GXS1* (b) expression in *S. cerevisiae* strains (see Table 1) cultivated in 2 % glucose. Northern blots were probed with a *GXF1*- or *GXS1*-specific probe, and subsequently with a *PDA1*-specific probe as a loading control.

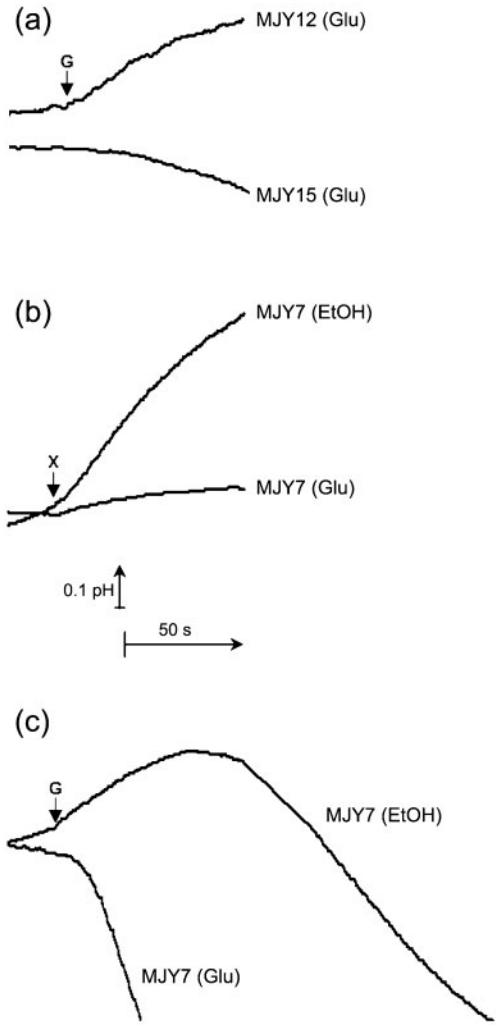

Fig. 6. Evaluation of Gxs1 symport activity in various strains and growth conditions. The effect on extracellular pH is shown of xylose (X) or glucose (G) added to a final concentration of 6.7 mM to aqueous cell suspensions of various *S. cerevisiae* strains: (a) MJY12 and MJY15 cultivated in 2% glucose (Glu); (b) and (c) MJY7 cultivated in 1% ethanol (EtOH) or 2% glucose (Glu), as indicated. The arrows indicate the time of sugar addition.

and *GXS1* mRNA levels. *GXF1* mRNA levels were found to be unaltered upon co-expression with other transporters (Fig. 5a) in the MJY1, MJY5 and MJ15 strains. However, *GXS1* mRNA was clearly abundant in MJY2, MJY3 and MJY12, where the gene encodes the only glucose/xylose transport system present, and below the detection limit in MJY5, MJY15 and MJY16 co-expressing Gxf1p, and in MJY7 and MJY9, where the native *HXT* transporter genes are present (Fig. 5b). A similar effect was observed for the expression of the Gxs1–GFP fusion protein: in MJY6, where it is the sole transporter present, the respective mRNA is abundant, whereas it is undetectable in strain MJY8, which differs from the former only by the presence of the Hxt proteins (Fig. 5b).

In agreement with these results, Gxs1p was virtually absent from the plasma membrane of strain MJY5 (Fig. 4). In contrast, the amounts of Gxf1 protein in the plasma membrane of glucose-grown cells were similar in strains MJY1 and MJY5, as expected from the observations concerning *GXF1* mRNA in these strains (Figs 4 and 5).

A simple explanation of all our observations would be a sharp drop in the copy number of the plasmid carrying the *GXS1* gene, caused by the fact that Gxs1 activity becomes dispensable for the cell when passive glucose transporters are present in high-glucose medium. Indeed, in strain MJY5 we detected that although the plasmid copy number did not decrease, as judged by a Southern blot experiment designed to quantify plasmid-borne copies of the *LEU2* gene, many plasmids seemed to have lost the *GXS1* gene (results not shown). However, this does not explain the decrease in *GXS1* mRNA levels in strain MJY7 (*GXS1*+*HXT*s). This strain exhibited low *GXS1* mRNA levels (Fig. 5) and virtually no symport activity (Fig. 6b, c) when cultivated in minimal medium with glucose. However, when MJY7 was cultivated in ethanol medium, symport activity was found to be present (Fig. 6b, c). In ethanol-grown cells of MJY7, proton movements elicited by the addition of glucose to an aqueous cell suspension suggest that both passive and active transport systems were operating simultaneously (Fig. 6c): the initial, transient alkalinization caused by the proton influx associated with symport activity was soon counteracted by the acidification due to proton efflux resulting from the activation of the plasma membrane ATPase. This could mean that glucose uptake rates increased considerably, in comparison with the situation in which the low-capacity Gxs1p was the only transporter present and no acidification was observed (results not shown). These results favour the hypothesis that the symporter co-exists with the Hxt facilitators at the plasma membrane of strain MJY7 in ethanol-grown but not in glucose-grown cells. This was confirmed by Northern blotting (see Fig. 5 and time zero in Fig. 7) and by quantitative real-time RT-PCR (not shown), both of which indicated that *GXS1* mRNA is present in ethanol-grown cells of strain MJY7, while it is almost undetectable in glucose-grown cells of the same strain. In view of these data, there seem to be two prerequisites for the reduction in the amount of *GXS1* mRNA: the presence of a high-capacity glucose facilitator in the plasma membrane, to bring about vigorous glucose uptake, and the presence of glucose in the growth medium.

Glucose affects *GXS1* mRNA stability

The mRNA level for a specific gene at a given time is the net result of its synthesis and its degradation rates. We considered it unlikely that glucose would affect the transcription of *GXS1*, because two strong, constitutive *S. cerevisiae* promoters drive the constructs used in this work. Therefore, the hypothesis was put forward that the increase in glucose influx derived from the presence of a glucose

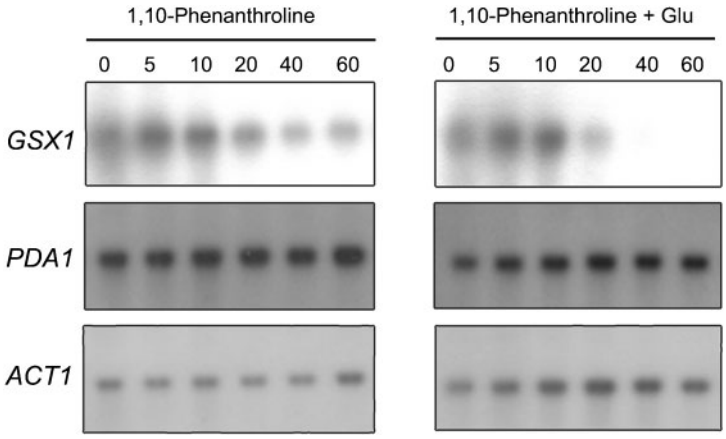

Fig. 7. Effect of glucose on *GXS1* mRNA stability. Northern blot analysis is shown of *GXS1* mRNA decay in ethanol-grown cells of *S. cerevisiae* MJY7. Time zero corresponds to cells collected immediately after a 1,10-phenanthroline pulse, with or without 2% glucose (Glu). The other lanes correspond to samples taken at different time points after the addition of 1,10-phenanthroline, as indicated. Northern blots were probed with a *GXS1*-specific probe, and subsequently with *PDA1*- and *ACT1*-specific probes as loading controls.

facilitator protein(s), rather than repression of transcription, would have a negative effect on mRNA stability. Actually, recent evidence in yeast suggests that mechanisms affecting mRNA turnover are quite often involved in the regulation of transcript levels as a response to the environment (Andrade *et al.*, 2005; Kresnowati *et al.*, 2006).

We sought to determine whether *GXS1* mRNA stability was affected by glucose. This was accomplished in experiments in which *de novo* mRNA synthesis was stopped by the addition of 1,10-phenanthroline, a compound often used as a transcription inhibitor in the determination of the half-life of mRNAs (Parker *et al.*, 1991). *S. cerevisiae* MJY7 (*GXS1*+*HXT*s) was first cultivated in ethanol medium. In two parallel experiments, 1,10-phenanthroline was subsequently added to the cells alone or simultaneously with glucose. Samples were collected at time zero and periodically after the addition of inhibitor, and total RNA was isolated. *GXS1*, as well as *PDA1* and *ACT1* (as loading controls), mRNA levels in the samples were analysed by Northern blotting. As expected, a decrease in *GXS1* mRNA levels was observed in all experiments, due to inhibition of transcription, but it remained above the detection level until 1 h after the addition of 1,10-phenanthroline. However, when glucose was added simultaneously with 1,10-phenanthroline, *GXS1* mRNA levels could no longer be detected after 40 min (Fig. 7), strongly suggesting that glucose accelerated *GXS1* mRNA decay.

DISCUSSION

We investigated the expression in *S. cerevisiae* of two *C. intermedia* glucose/xylose transporters, Gxf1p and Gxs1p, which display markedly different behaviours in the heterologous environment. Our findings show that both proteins are produced abundantly in *S. cerevisiae* and that both are correctly targeted to the plasma membrane, even when overexpressed. In fact, SDS-PAGE analysis of plasma membrane preparations of *S. cerevisiae* transformants expressing each heterologous transporter shows that Gxf1p and Gxs1p are produced in similar amounts, and that they represent a major fraction of the plasma membrane proteins in strains MJY1 (expressing Gxf1p) and MJY2 (expressing Gxs1p). However, while Gxf1p seems to be fully functional in *S. cerevisiae*, the symporter protein Gxs1 exhibits very low sugar transport activity compared with that of the gene donor strain of *C. intermedia*. This observation, together with an apparent phylogenetic relationship between Gxs1p and the glucose 'sensors' Snf3 and Rgt2 from *S. cerevisiae*, raises the question of whether this protein functions in its native environment mainly as a sensor and not as a transporter. Two observations argue against this hypothesis: (i) Gxs1p is a highly abundant protein in the plasma membrane of *C. intermedia* under inducible conditions (Leandro *et al.*, 2006); and (ii) *S. cerevisiae* transformants expressing Gxs1p as the sole transporter exhibit glucose and xylose proton symport activity and are able to grow on glucose, though they hardly grow on xylose.

It is of note that a xylose (high-affinity) transporter gene recently isolated from the fungus *Trichoderma reesei* (*Hypocrea jecorina*) is also unable to support growth of *S. cerevisiae* with xylose as the sole carbon and energy source. However, growth on xylose improved after prolonged culture on xylose medium supplemented with maltose as a consequence of mutations that occurred spontaneously in the host strain (Saloheimo *et al.*, 2007). A similar observation has been described for the expression of the mammalian glucose transporters GLUT1 and GLUT4 in *S. cerevisiae* (Wieczorke *et al.*, 2003). In this case, mutations in the host strain were required for correct targeting of the protein to the plasma membrane. The mechanism(s) by which host mutations improve functional expression of heterologous membrane proteins are still unclear, but one possibility is the activation of auxiliary factors. It has been suggested that such factors could help folding of permeases, form complexes with permeases to activate them, or have a regulatory role in the activity of certain transporters (Saloheimo *et al.*, 2007).

Another possible explanation for the reduced activity of Gxs1p in *S. cerevisiae* could be related to the interactions

between the protein and the surrounding lipids in the membrane. The lipid composition of the plasma membrane may vary considerably as a function of the growth conditions and the yeast (Hunter & Rose, 1971; Opekarová & Tanner, 2003). Should such differences occur between *S. cerevisiae* and *C. intermedia*, they could affect the biochemical properties of heterologously expressed integral membrane proteins from *C. intermedia*. For example, a change in the hydrophobic thickness of the lipid bilayer (defined by the lengths of the fatty acyl chains in the lipid components) would result in significant changes in the conformation of membrane proteins, which are likely to cause changes in activity (Opekarová & Tanner, 2003; Lee, 2004).

Based on available knowledge, it is not possible to outline general methods to improve functionality of heterologous membrane proteins in *S. cerevisiae*, since different proteins seem to require specific and distinct conditions to be correctly targeted to the plasma membrane and/or display their full activity (Wieczorke *et al.*, 2003; Flegelova *et al.*, 2006). This is illustrated by our observations of the deleterious effect on Gxs1p expression of a mutation selected for improving functional expression of the mammalian GLUT1 transporter in *S. cerevisiae* (results not shown). In the work described here, having excluded the possibility that Gxs1p is deficiently produced or incorrectly targeted, we postulate that poor activity could be due to a more subtle shortcoming of the environment in the heterologous host, such as the absence of an auxiliary protein or a different membrane composition.

In the course of our attempts to improve functional expression of Gxs1p in *S. cerevisiae* we found that co-expression of glucose/xylose facilitators, such as Hxts, with Gxs1p strongly reduced *GXS1* mRNA levels and, consequently, symport activity. This occurred in glucose-grown but not in ethanol-grown cells, suggesting that the reduction in the levels of the *GXS1* transcript relates to the enhancement of glucose influx and not to a specific interaction between transporters. In this respect, the decrease in *GXS1* mRNA levels resembles many other glucose-triggered cellular responses that operate mainly at the transcription level (Gancedo, 1998; Rolland *et al.*, 2002). However, the glucose-responsive decrease in *GXS1* transcript levels was rather unexpected, because constitutive glucose repression-insensitive *S. cerevisiae* promoters drive the expression of the respective cDNA in all our strains. The possibility that glucose affects *GXS1* mRNA stability, instead of the rate of transcription, was investigated, and we found evidence for a decreased lifetime of *GXS1* mRNA in the presence of glucose. The mechanism affecting *GXS1* expression might also explain the failure of attempts to co-express pairs of Hxt proteins with different kinetic properties, driven by constitutive promoters (E. Boles, personal communication). Many different genes in *S. cerevisiae* have been shown to be regulated at the level of mRNA stability in response to glucose, e.g. *SUC2* (encoding invertase; Cereghino & Scheffler, 1996) and *JEN1* (encoding a lactate transporter; Andrade *et al.*, 2005). In addition, a recent whole-genome analysis of the glucose response in *S. cerevisiae* has also revealed that large sets of genes involved in the tricarboxylic acid cycle and storage carbohydrate metabolism are downregulated by glucose at the level of mRNA stability (Kresnowati *et al.*, 2006).

The model proposed for mRNA turnover in yeast comprises three sequential steps: shortening of the poly(A) tail, decapping, and 5′–3′ exonucleolytic degradation (Prieto *et al.*, 2000). It has been proposed that glucose causes the 5′ cap of the mRNA to become more accessible to decapping, and that competition occurs between the decapping machinery and the assembly of the translation initiation complex for the 5′ cap region (Scheffler *et al.*, 1998; de la Cruz *et al.*, 2002). The outcome of this competition seems to depend on both the sequence and the length of the 5′ untranslated region (UTR). The presence of a long poly(A) tail may also affect this competition in favour of translational initiation and mRNA stabilization (Prieto *et al.*, 2000). Moreover, conserved 3′ UTR motifs have been identified in glucose-downregulated genes that may be involved in specific degradation (Kresnowati *et al.*, 2006).

In the case of *GXS1* mRNA, it is unlikely that either 5′ or 3′ UTR signals mediate glucose-accelerated degradation, because the UTRs present in this mRNA (namely the 5′ UTR from the *HXT7* and *PGK1* genes and the 3′ UTR from the *PGK1* gene) do not mediate glucose-accelerated mRNA degradation in either their natural contexts or other fusion constructs (Kingsman *et al.*, 1990; Hauf *et al.*, 2000; Hamacher *et al.*, 2002). It follows that such a signal is most probably located in the coding region of the *GXS1* gene. The possibility that destabilization of *GXS1* mRNA in the presence of high glucose concentrations also occurs in *C. intermedia* to reinforce transcriptional repression, possibly by a mechanism similar to that operating in *S. cerevisiae* transformants, cannot be discarded. We have evidence supporting conservation of regulatory mechanisms in *S. cerevisiae* and *C. intermedia*, at least at the transcriptional level: the *GXF1* (facilitator) gene is very efficiently expressed in *S. cerevisiae* from its own (*C. intermedia*) promoter and, in addition, it seems to be similarly regulated by the carbon source (Leandro *et al.*, 2006), i.e. it is poorly expressed in the absence of glucose or xylose (data not shown).

In different yeasts displaying multiple transport systems for glucose or fructose, the expression of those sugar transporters is stringently regulated according to the needs of the cell. The expression profile is determined by the external sugar concentration, and is usually linked to the mechanistic and kinetic properties of the various transporters. In particular, active transport systems are often prevented from operating unless they are strictly required (Spencer-Martins & van Uden, 1985; Cason *et al.*, 1986; Verma *et al.*, 1987; Lucas & van Uden, 1988; Nobre *et al.*, 1999; Gonçalves *et al.*, 2000; Gárdonyi *et al.*, 2003b). In *S.*

cerevisiae, several regulatory mechanisms have been shown to prevent inappropriate expression of sugar transporters. Both transcriptional repression and the specific removal from the membrane and degradation of transporters can be involved (Krampe *et al.*, 1998; Kresnowati *et al.*, 2006). We did not find any evidence for the specific degradation of Gxs1p in the presence of high glucose concentrations, even with a functioning facilitated glucose transport system (results not shown). Our work suggests that regulation of mRNA stability might be an additional, ubiquitous mechanism used by yeast cells to enforce downregulation of high-affinity transporters, in particular those that may pose an unnecessary energetic burden on the cells by operating when sugar is plentiful and fermentation is the preferred metabolic route. A comparative study of the functional expression of the two *C. intermedia* transporters in *S. cerevisiae* constitutes an attractive model to investigate important bottlenecks in the heterologous expression of sugar transporters.

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