

Rewiring yeast sugar transporter preference through modifying a conserved protein motif

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Edited by Lonnie O. Ingram, University of Florida, Gainesville, FL, and approved November 21, 2013 (received for review June 24, 2013)

Utilization of exogenous sugars found in lignocellulosic biomass hydrolysates, such as xylose, must be improved before yeast can serve as an efficient biofuel and biochemical production platform. In particular, the first step in this process, the molecular transport of xylose into the cell, can serve as a significant flux bottleneck and is highly inhibited by other sugars. Here we demonstrate that sugar transport preference and kinetics can be rewired through the programming of a sequence motif of the general form G-G/F-XXX-G found in the first transmembrane span. By evaluating 46 different heterologously expressed transporters, we find that this motif is conserved among functional transporters and highly enriched in transporters that confer growth on xylose. Through saturation mutagenesis and subsequent rational mutagenesis, four transporter mutants unable to confer growth on glucose but able to sustain growth on xylose were engineered. Specifically, *Candida intermedia gxs1* Phe³⁸Ile³⁹Met⁴⁰, *Scheffersomyces stipitis rgt2* Phe³⁸ and Met⁴⁰, and *Saccharomyces cerevisiae hxt7* Ile³⁹Met⁴⁰Met³⁴⁰ all exhibit this phenotype. In these cases, primary hexose transporters were rewired into xylose transporters. These xylose transporters nevertheless remained inhibited by glucose. Furthermore, in the course of identifying this motif, novel wild-type transporters with superior monosaccharide growth profiles were discovered, namely *S. stipitis RGT2* and *Debaryomyces hansenii 2D01474*. These findings build toward the engineering of efficient pentose utilization in yeast and provide a blueprint for reprogramming transporter properties.

metabolic engineering | xylose metabolism | protein engineering | transporter engineering

Molecular transporter proteins facilitate monosaccharide uptake and serve as the first step in catabolic metabolism. In this capacity, the preferences, regulation, and kinetics of these transporters ultimately dictate total carbon flux (1–3); and optimization of intracellular catabolic pathways only increases the degree to which transport exerts control over metabolic flux (4, 5). Thus, monosaccharide transport profiles and rates are important design criteria and a driving force to enable metabolic engineering advances, ultimately resulting in a biorefinery concept whereby biomass is converted via microbes into a diverse set of molecules (6–10). Among possible host organisms, *Saccharomyces cerevisiae* is an emerging industrial organism with well-developed genetic tools and established industrial processes and track record (11–16). However, *S. cerevisiae* lacks an endogenous xylose catabolic pathway and thus is unable to natively use the second most abundant sugar in lignocellulosic biomass. Decades of research have been focused on improving xylose catabolic pathways in recombinant *S. cerevisiae* (17–22), but less work has been focused on the first committed step of the process—xylose transport, an outstanding limitation in the efficient conversion of lignocellulosic sugars (23, 24).

In *S. cerevisiae*, monosaccharide uptake is mediated by transporters belonging to the major facilitator superfamily (MFS) (25, 26), a ubiquitous group of proteins found across species (27). The predominant transporters in yeast are members of the HXT family (28) and are marked by efficient hexose transport (29) with lower affinities to xylose thus contributing to diauxic

growth and flux limitation when attempting pentose utilization in recombinant *S. cerevisiae* (30). Previous efforts have attempted to identify heterologous transporters with a higher affinity for xylose over glucose (31–36). However, the vast majority of these transporters are either nonfunctional, not efficient, or not xylose specific (24, 37). Furthermore, nearly all known wild-type transporters that enable growth on xylose in yeast confer higher growth rates on glucose than on xylose (24, 37). As an alternative to bioprospecting, we have previously reported that xylose affinity and exponential growth rates on xylose can be improved via directed evolution of *Candida intermedia* glucose-xylose symporter 1 (*GXS1*) and *Scheffersomyces stipitis* xylose uptake 3 (*XUT3*) (38). These results demonstrated that mutations at specific residues (e.g., Phe⁴⁰ in *C. intermedia* *GXS1*) can have a significant impact on the carbohydrate selectivity of these MFS transporters. The fact that single amino acid substitutions can have such a significant impact on transport phenotype (38–40) indicates how simple homology based searches can be ineffective at identifying efficient xylose transporters (35, 36). However, evidence of natural xylose exclusivity is seen in the *Escherichia coli xylE* transporter that has recently been crystallized (41). The sequence-function flexibility of MFS transporters potentiates the capability to rewire hexose transporters from being glucose favoring, xylose permissive into being xylose-exclusive transporters.

In this work, we report on the discovery of a conserved Gly³⁶-Gly³⁷-Val³⁸-Leu³⁹-Phe⁴⁰-Gly⁴¹ motif surrounding the previously identified Phe⁴⁰ residue of *C. intermedia* *GXS1* that controls transporter efficiency and selectivity. By evaluating 46 different heterologously expressed transporters, we find that this motif is conserved among functional transporters and highly enriched in transporters that confer growth on xylose, taking the general form G-G/F-XXX-G. We conduct saturation mutagenesis on Val³⁸, Leu³⁹, and Phe⁴⁰ within the variable region of this motif in *C. intermedia* *GXS1* to demonstrate control of sugar selectivity. Next, we combine xylose-favoring mutations to create a unique mutant version of *gxs1* that transports xylose, but not glucose.

Significance

The quest for an optimal xylose pathway in yeast is of utmost importance along the way to realizing the potential of lignocellulosic biomass conversion into fuels and chemicals. An often-overlooked aspect of this catabolic pathway is the molecular transporter of this sugar. Here we demonstrate that sugar transport preference and kinetics can be rewired through the programming of a specific sequence motif. The result is a study to rationally alter the sugar preference of a protein through defined sequence-level modifications. In these cases, primary hexose transporters were rewired into xylose transporters.

Author contributions: E.M.Y. and H.S.A. designed research; E.M.Y., A.T., H.B., and C.S. performed research; E.M.Y. and H.S.A. analyzed data; and E.M.Y. and H.S.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1311970111/-DCSupplemental.

Finally, we demonstrate the importance of this motif in the capacity to rewire the sugar preference of other hexose transporters including *S. cerevisiae* hexose transporter 7 (HXT7) and *S. stipitis* glucose transporter/sensor (RGT2, similar to *S. cerevisiae* RGT2). This work serves to increase our understanding of the structure–function relationships for molecular transporter engineering and demonstrates complete rewiring of hexose transporters into transporters that prefer xylose as a substrate.

Results

Identification of the G-G/F-XXX-G Motif That Controls Sugar Transport Preference. Previously, we reported on single amino acid substitutions at Phe⁴⁰ in transmembrane span 1 (TMS1) that can alter monosaccharide transport profiles of *C. intermedia* GXS1 (38). The potency of this residue as well as its proximity to the outer pore of the transporter suggested it could be part of an important contact and recognition site for monosaccharides. A multiple sequence alignment of 26 previously cloned transporters (36) indicates that Phe⁴⁰ was indeed part of a highly conserved glycine-rich motif of the form G-G/F-XXX-G, where X represents a variable, but usually nonpolar amino acid residue. In *C. intermedia* GXS1, the wild-type motif is Gly³⁶Gly³⁷Val³⁸Leu³⁹Phe⁴⁰Gly⁴¹. The high conservation of this motif suggested it could be responsible for xylose uptake, transporter efficiency, and monosaccharide selectivity. To further corroborate this hypothesis, an additional 20 putative transporters were identified using a BLAST search seeded with transporters identified in our prior study and functionally characterized in *S. cerevisiae* EX.12, a recombinant strain lacking endogenous monosaccharide transporters (SI Appendix, Fig. S1 and Table S1) (26, 38). The vast majority of these transporters were functional and all possessed a similar motif. Among these transporters, *Debaryomyces hansenii* 2D01474 confers much faster growth on xylose than on glucose and *S. stipitis* RGT2 confers the fastest growth on xylose of all of the *S. stipitis*-derived transporters in this study.

Following the functional characterization, we correlated motif sequence and the resulting transporter carbon source profile. Four major phenotypic classifications were made: (i) transporters that failed to function heterologously ($\mu_{\text{all}} = 0$), (ii) transporters that conferred growth on a hexose but not xylose ($\mu_X = 0$), (iii) transporters that conferred growth on xylose but not as fast as glucose ($\mu_X < \mu_G$), and (iv) transporters that conferred a higher growth rate on xylose than on glucose ($\mu_X > \mu_G$). Fig. 1A displays the relative proportions of each of these

classifications in the group of 46 transporters studied. To characterize the sequence, four major motif classifications were made: (i) a full G-G/F-XXXG motif, (ii) a related S-G-XXXG motif, (iii) a motif unrelated to the glycine-rich motif, and (iv) the lack of homology to other transporters at both the motif and surrounding residues. Fig. 1B depicts the distribution of the four sequence motif classifications within the four phenotypic classifications. Strikingly, there is a clear enrichment of the G-G/F-XXXG motif among the functional transporters that enable high xylose transport rates. In fact, this motif is exclusively seen in phenotype class iv where $\mu_X > \mu_G$. The enrichment and convergence of the variable residues within the motif is displayed in Fig. 1C. It should be noted that the consensus sequence from this analysis appears to be G-G/F-XX-F-G. However, variations at the consensus F residue led to the discovery of the motif, therefore this position was considered variable. Fig. 1C highlights the strong correlation between sequence motif and xylose transport function and suggests an important role of TMS1 on sugar recognition.

Identification of Potentiating Variable Residues Within the G-G/F-X-X-G Motif. To examine the role of the variable region, we performed complete saturation mutagenesis for each of the three residues (Val³⁸, Leu³⁹, and Phe⁴⁰) in *C. intermedia* GXS1 and evaluated the impact on carbon source growth profile as measured by growth rate. Our previous studies demonstrate that growth rate in this test strain is a good surrogate for transporter kinetics (36, 38). Specifically, the fractional change in the growth rate of *S. cerevisiae* EX.12 on glucose, xylose, galactose, fructose, or mannose as the sole carbon source was evaluated compared with the wild-type transporter. The impact of each residue can be classified as having no change, altered efficiency, altered selectivity, or a combination of the three (Fig. 2). For creating xylose-specific transporters, the goal is to identify mutations that attenuate hexose growth while either amplifying or maintaining xylose growth.

Members of the *C. intermedia* gxs1 Val³⁸ saturation library (Fig. 3A) display differential exponential growth rates with the most significant one being the Phe³⁸ substitution. This mutant confers a selectivity phenotype that almost completely attenuates glucose exponential growth rate while amplifying exponential xylose growth rate by 50%. Other substitutions that confer desirable selectivity phenotypes are Asp³⁸, Cys³⁸, Gly³⁸, and His³⁸. All of these affect the growth profile in different patterns, but none as significantly as Phe³⁸. Three substitutions, Ile³⁸, Leu³⁸,

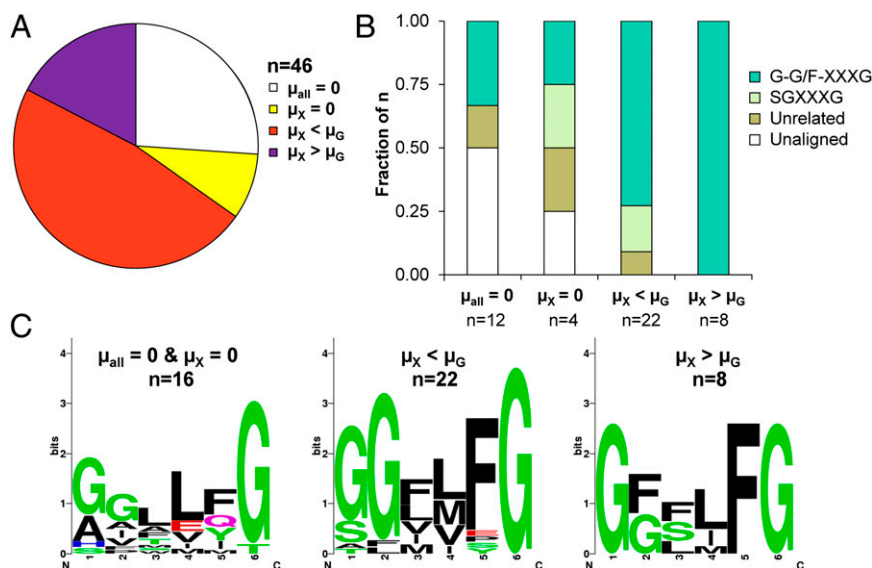


Fig. 1. Sequence categorization and phenotypic classification of native and heterologous transporters. (A) The distribution of phenotypic classes for all 46 transporters. (B) The distribution of each sequence category present in each phenotypic class. Transporters containing the conserved motif are enriched in the phenotypic classes that confer growth on xylose. (C) Weblogs of the phenotypic classes illustrate enrichment of the G-G/F-XXXG motif in TMS1. $\mu_{\text{all}} = 0$ represents no growth in the five carbon sources tested; $\mu_X = 0$ represents growth on hexoses but not xylose, $\mu_X < \mu_G$ represents growth on xylose is less than that on glucose, and $\mu_X > \mu_G$ represents growth on xylose is greater than that on glucose.

growth profile while severely attenuate glucose growth. We next performed further characterization of the best mutant, *gxs1* Phe³⁸Ile³⁹Met⁴⁰. First, to assay transport capacity, high cell density fermentations with xylose and glucose were performed (Fig. 5A and B). The Phe³⁸Ile³⁹Met⁴⁰ triple mutant displayed no appreciable glucose uptake whereas xylose uptake has become more efficient compared with the wild-type GXS1. These results display a rewiring of the sugar uptake ratio. However, despite eradicating glucose transport capacity, glucose at levels of 5 g/L still inhibit xylose growth (Fig. 5C). This finding is corroborated by high cell density cofermentations (SI Appendix, Figs. S2 and S3).

Second, radiolabeled xylose uptake experiments were performed to quantify the improvement of transport kinetics in the Phe³⁸Ile³⁹Met⁴⁰ triple mutant. The improvements in xylose utilization observed at high cell density culturing were mainly due to a doubling in V_{max} (Fig. 5D). An increased K_M was observed as well (Fig. 5E), a phenotype observed in previous efforts to engineer this transporter (38). Nevertheless, the binding affinity is still quite high for practical culturing at a value corresponding to around 0.1 g/L (SI Appendix, Table S2). These kinetics experiments were also performed in the presence of glucose and no radiolabeled xylose uptake was detected indicating that although glucose cannot pass through the transporter, it can still bind and inhibit xylose uptake. Hence, binding must occur at a different residue.

The G-G/F-XXXG Motif Can Be Used to Rewire Other Transporters.

Finally, we used the conserved G-G/F-XXXG motif to rewire the sugar preference of other predominately hexose transporters. Specifically, two transporters, *S. stipitis* RGT2 and *S. cerevisiae* HXT7, were selected based on evolutionary distance from GXS1. *S. stipitis* RGT2 is closely related to *C. intermedia* GXS1, whereas the native HXT transporters are more distant (SI Appendix, Figs. S4 and S5). First, we investigated the impact of rewiring the closely related transporter, *S. stipitis* RGT2. This transporter contains a G³⁶G³⁷I³⁸L³⁹F⁴⁰G⁴¹ motif and we characterized two separate point mutations, Phe³⁸ and Met⁴⁰. In both cases, glucose growth has been completely attenuated (Fig. 6). Most striking is the Met⁴⁰ mutation, which eliminates growth on all carbon sources but xylose and galactose. By modifying the motif in RGT2, we generated two additional mutant proteins that transport xylose but not glucose.

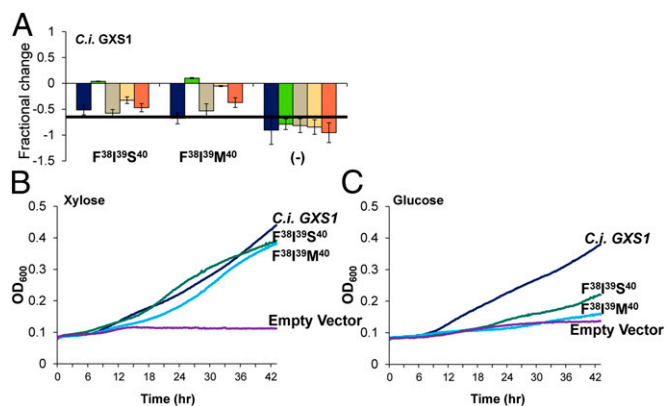


Fig. 4. Growth characterization of *C. intermedia* *gxs1* triple mutants. (A) Fractional change from wild type for the two triple mutants and an empty vector control. A bar chart of the exponential growth rates from which fractional change was calculated can be found in SI Appendix. Error bars were calculated using the sum of least squares method. (B) Average growth curves on xylose based on OD₆₀₀. (C) Average growth curves on glucose based on OD₆₀₀. Calculated exponential growth rates are shown in SI Appendix, Fig. S7.

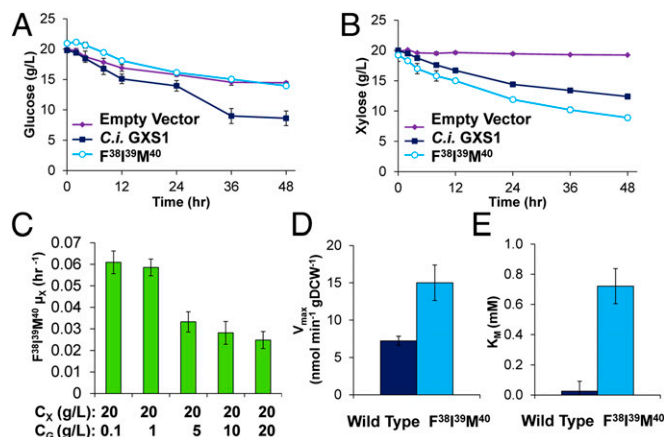


Fig. 5. Further characterization of *C. intermedia* *gxs1* Phe³⁸Ile³⁹Met⁴⁰ triple mutant. (A) Glucose uptake at high cell density for *S. cerevisiae* EX.12 expressing wild type, Phe³⁸Ile³⁹Met⁴⁰, and empty vector. (B) Xylose uptake at high cell density for *S. cerevisiae* EX.12 expressing wild type, Phe³⁸Ile³⁹Met⁴⁰, and empty vector. (C) Inhibition of the growth rate on xylose with increasing glucose concentration. (D) V_{max} of both the wild type and the mutant. (E) K_M of both the wild type and triple mutant. (A–E) Error is based on SD of biological replicates.

Second, we evaluated the potential to rewire *S. cerevisiae* HXT7, a more distantly related protein yet able to efficiently transport hexoses and xylose in yeast (32, 42). Given the proficiency of hexose transport by this protein, rewiring to attenuate growth on hexoses presents a greater challenge. The native motif within *S. cerevisiae* HXT7 is G³⁶G³⁷F³⁸V³⁹F⁴⁰G⁴¹. We initially evaluated two double mutations to this motif: Ile³⁹Met⁴⁰ and His³⁹Met⁴⁰. Fig. 7 demonstrates that the Ile³⁹Met⁴⁰ double mutant amplified xylose exponential growth and attenuated growth on all hexoses save glucose whereas the His³⁹Met⁴⁰ double mutant attenuated glucose growth yet severely attenuated xylose exponential growth. Previous studies have indicated that mutations at Asp³⁴⁰ can eliminate glucose transport (39) in HXT7 and we verify here that transport of nearly all monosaccharides is severely attenuated with this mutation (Fig. 7). Coupling the Met³⁴⁰ mutation with the Ile³⁹Met⁴⁰ double mutant resulted in robust growth on xylose while maintaining the inability to transport glucose. With this triple mutant, a robust hexose transporter was converted to a xylose transporter unable to support growth on glucose.

Discussion

We identified a short, six-residue motif of the form G-G/F-XXXG in TMS1 that exerts control over selectivity and efficiency of monosaccharide transport of MFS family transporters. This motif is conserved among functional transporters and highly enriched in transporters that confer growth on xylose. Altering the composition of the variable region changes the sugar uptake profiles of these transporters and can thus be used to rewire transporter function. Altering the residues in this domain can eliminate glucose transport while retaining xylose transport, a major step forward for molecular transporter engineering. In doing so, we create several transporter mutants that support the transport of xylose and not glucose.

Hydrophobic, nonpolar, and moderate-to-large-size residues often attenuated glucose compared with xylose. Amino acids such as Phe, Ile, Ser, and Met were among the most effective substitutions that differentially amplified xylose growth rate. Although many of these residues are found naturally in wild-type motif sequences (Fig. 2), the most effective combinations found here (particularly Phe³⁸Ile³⁹Met⁴⁰) are not found naturally (SI Appendix, Table S3). Hypotheses concerning transporter

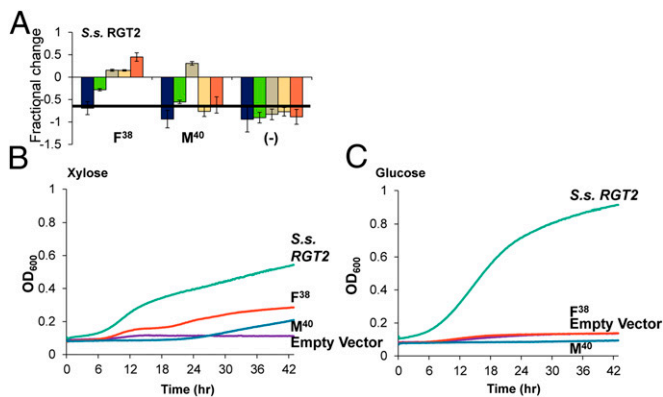


Fig. 6. Growth characterization of *S. stipitis* RGT2 and mutants. (A) Fractional change from wild type for the two single mutants and an empty vector control. Error bars were calculated using the sum of least squares method. A bar chart of the exponential growth rates from which fractional change was calculated can be found in *SI Appendix*. (B) Average growth curves on xylose based on OD₆₀₀. (C) Average growth curves on glucose based on OD₆₀₀. Calculated maximum exponential growth rates are shown in *SI Appendix*, Fig. S7.

substrate recognition and transport mechanism may be formed based on these results. The advantage of large and nonpolar residues suggests that glucose growth attenuation is due to steric exclusion. The larger side chains would physically restrict the size of the pore, allowing the smaller xylose molecule to bind and traverse more efficiently than larger hexoses. A similar hypothesis has been proposed to explain an observed correlation between amino acid size and transporter function for glucose (43). This hypothesis is supported by the crystal structure of a related MFS transporter, *E. coli* *xylE* (41). Based on the structure, *E. coli* *xylE* Phe²⁴, an analogous residue to *C. intermedia* *gxs1* Phe⁴⁰, appears to interact with sugars as they pass through the pore. Unfortunately, *E. coli* *xylE* is too dissimilar from yeast MFS transporters to enable structure prediction, yet this evidence suggests that this residue appears to play a role in all MFS sugar transporters. Further crystal structures of more closely related transporters will greatly enhance the understanding of the yeast MFS sugar transporter mechanism.

Before this study, prior evidence has pointed to the possibility of xylose exclusivity. Transporters from *Neurospora crassa* and *S. stipitis* were found to be exclusive for xylose in uptake assays (35), but are unable to support robust growth of recombinant *S. cerevisiae* on xylose. The *E. coli* *xylE* transporter is xylose specific when expressed in its native host (44), but is inhibited by glucose and remains nonfunctional in *S. cerevisiae* despite attempts at directed evolution. This work has demonstrated a defined transporter engineering approach that is able to effectively eliminate glucose transport while amplifying xylose transport and supporting robust xylose growth. The mutants generated in this study demonstrate this desirable phenotype and provide evidence that the G-G/F-XXXG motif controls the transport phenotype in a large number of MFS transport proteins.

It is also important to note that altering this motif in *C. intermedia* *GXS1* not only had an impact on glucose uptake, but also had an impact on the kinetics of xylose uptake. Specifically, the K_m for xylose was significantly increased compared with wild type, indicating that exclusion of glucose was obtained at the expense of a reduced affinity for xylose. Nevertheless, the affinity for xylose remains sufficiently high for nearly all fermentation conditions ($K_M = 0.721 \pm 0.116$ mM, or ~ 0.1 g/L), and was partially compensated by a doubling in V_{max} (Fig. 5). This result suggests a complex set of interactions between the transporter and sugar substrate, and is similar to other mutants of *C. intermedia* *GXS1* (38).

In the course of identifying and validating this motif, several previously uncharacterized native and heterologous transporters were identified and shown to possess previously unreported phenotypes (*SI Appendix*, Fig. S6). The transporter *D. hansenii* 2D01474 can natively support high exponential growth on xylose compared with glucose. The transporter *S. stipitis* RGT2 confers the fastest growth rate on xylose over any ORF cloned from *S. stipitis* and thus may be a member of the long sought-after xylose transporter suite in the efficient xylose-fermenting organism *S. stipitis*. Both of these transporters are closely related to *C. intermedia* *GXS1* (*SI Appendix*, Fig. S5) and may present a unique class of related transporters that make excellent starting scaffolds for engineering exclusive xylose uptake. Of the remaining ORFs studied here, one group (*D. hansenii* 2E01166, *D. hansenii* 2B05060, *S. cerevisiae* *STL1*, and *S. stipitis* *AUT1*) confers much higher exponential growth rates on galactose than any other sugar tested. This hexose transport profile is indicative of the potential for L-arabinose transport, as the galactose transporter *S. cerevisiae* *GAL2* is one of the few transporters able to facilitate L-arabinose (45). This correlation is likely due to the similar stereochemistry between L-arabinose and galactose.

In summary, this work describes the discovery of a conserved G-G/F-XXXG motif and an engineering approach to modify this motif. This motif allowed for the rewiring of several transporters and yielded the mutant transporters *C. intermedia* *gxs1* Phe³⁸Ile³⁹Met⁴⁰, *S. stipitis* *rgt2* Phe³⁸ and Met⁴⁰, and *S. cerevisiae* *hxt7* Ile³⁹Met⁴⁰Met³⁴⁰ that do not transport glucose yet support *S. cerevisiae* *EX.12* growth on xylose. These MFS transporters are channels and thus a substrate molecule interacts with many residues during transport, yet the few residues in this motif appear to have a great deal of influence over glucose transport attenuation and xylose transport amplification. Thus, this study provides further insight into the residues responsible for monosaccharide transport in MFS proteins while establishing a platform for engineering a specific, efficient xylose transporter.

Materials and Methods

Strains, Media, and Plasmids. Molecular cloning and standard culturing techniques with *E. coli* *DH10B* were performed according to Sambrook (46). *S. cerevisiae* *EX.12* was used for all yeast experiments and was constructed as previously described (38). All transporters were cloned into p414-TEF, a standard yeast shuttle vector created by Mumberg et al. (47). Yeast synthetic complete media was used for culture and experimental growth media. Complete supplemented media without tryptophan was used

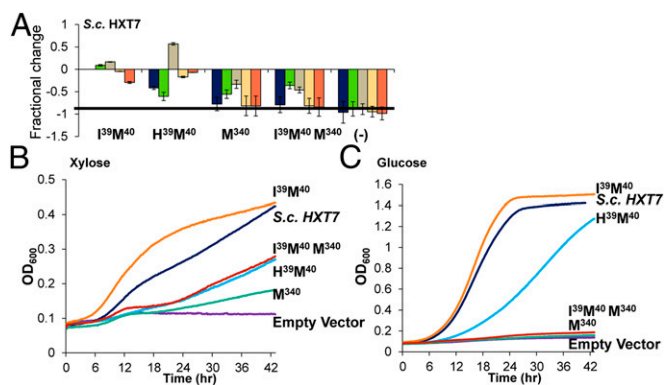


Fig. 7. Growth characterization of *S. cerevisiae* HXT7 and mutants. (A) Fractional change from wild type for the mutants and an empty vector control. Error bars were calculated using the sum of least squares method. A bar chart of the exponential growth rates from which fractional change was calculated can be found in *SI Appendix*. (B) Average growth curves on xylose based on OD₆₀₀. (C) Average growth curves on glucose based on OD₆₀₀. Calculated maximum exponential growth rates are shown in *SI Appendix*, Fig. S7.

when *S. cerevisiae* EX.12 was carrying a transporter. Carbon sources were provided at 20 g/L.

Transporter Cloning. Potential xylose transporters were identified from the literature and BLAST search. To obtain this list of 46, we combined 26 transporters from our previous survey of transporters (36) along with 20 additional transporters identified through homology search using *C. intermedia* GX51 and *S. cerevisiae* STL1 as a template. Details on cloning and transporter libraries are described in *SI Appendix*. Primers for cloning, saturation mutagenesis, and point mutations are listed in *SI Appendix, Tables S4, S5, and S6*, respectively.

Growth Rate Measurements. All exponential growth rates were measured and calculated according to the method previously described using a Bioscreen C (Growth Curves USA) and a MATLAB script (36, 38). Details of the measurements are provided in *SI Appendix*.

Fractional Change. Fractional change in the growth rate from wild type was calculated by taking the difference between the growth rates of the mutant and wild type over the growth rate of the wild type for each individual carbon

source. Error was propagated using the least squares method based on the SD in exponential growth rates of the mutant and the wild type.

High Cell Density Fermentation. High cell density experiments were conducted as previously described (38). Yeast cultures were suspended at OD₆₀₀ of 20 in 20 g/L glucose, 10 g/L glucose and 10 g/L xylose, or 20 g/L xylose. The supernatant concentration of xylose and/or glucose was measured using a YSI Life Sciences Bioanalyzer 7100MBS.

Radiolabeled Xylose Uptake. Uptake of ¹⁴C-labeled xylose was used to determine the Michaelis–Menten parameters for *C. intermedia* GX51 and the Phe³⁸Ile³⁹Met⁴⁰ triple mutant. The method was performed as previously described (38).

ACKNOWLEDGMENTS. This work was supported by National Science Foundation (NSF) Grant CBET-1067506 and E. Young is supported by an NSF Graduate Research Fellowship. We acknowledge Dr. Marvin Whiteley for providing laboratory space and equipment for the radiolabeled xylose uptake experiments and thank Dr. Eckhard Boles for providing *S. cerevisiae* EBY.VW4000.

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