APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 2005, p. 6465–6472 Vol. 71, No. 11
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.11.6465–6472.2005

Improvement of Galactose Uptake in *Saccharomyces cerevisiae* through Overexpression of Phosphoglucomutase: Example of Transcript Analysis as a Tool in Inverse Metabolic Engineering

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Received 4 April 2005/Accepted 10 June 2005

Through genome-wide transcript analysis of a reference strain and two recombinant *Saccharomyces cerevisiae* strains with different rates of galactose uptake, we obtained information about the global transcriptional response to metabolic engineering of the *GAL* gene regulatory network. One of the recombinant strains overexpressed the gene encoding the transcriptional activator Gal4, and in the other strain the genes encoding Gal80, Gal6, and Mig1, which are negative regulators of the *GAL* system, were deleted. Even though the galactose uptake rates were significantly different in the three strains, we surprisingly did not find any significant changes in the expression of the genes encoding the enzymes catalyzing the first steps of the pathway (i.e., the genes encoding Gal2, Gal1, Gal7, and Gal10). We did, however, find that *PGM2*, encoding the major isoenzyme of phosphoglucomutase, was slightly up-regulated in the two recombinant strains with higher galactose uptake rates. This indicated that *PGM2* is a target for overexpression in terms of increasing the flux through the Leloir pathway, and through overexpression of *PGM2* the galactose uptake rate could be increased by 70% compared to that of the reference strain. Based on our findings, we concluded that phosphoglucomutase plays a key role in controlling the flux through the Leloir pathway, probably due to increased conversion of glucose-1-phosphate to glucose-6-phosphate. This conclusion was supported by measurements of sugar phosphates, which showed that there were increased concentrations of glucose-6-phosphate, galactose-6-phosphate, and fructose-6-phosphate in the strain construct overexpressing *PGM2*.

In the yeast *Saccharomyces cerevisiae* the flux through the galactose utilization pathway is threefold lower than the rate of glucose utilization (35). From an industrial point of view galactose utilization is relevant due to the presence of galactose in several industrial media, such as cheese whey (see reference 43 for a review), molasses (40), and lignocellulose (53). Therefore, there is interest in increasing galactose utilization through the use of metabolic engineering (3, 30, 34). Besides the industrial relevance, the gene system involved in galactose metabolism in yeast (the *GAL* genes) has served as a eukaryotic model system for gene regulation and thus is one of the best-characterized systems of eukaryotic transcriptional control (18, 20). Moreover, the importance of the *S. cerevisiae GAL* system is further emphasized by its role as a model for the human hereditary disease galactosemia (42), which is caused by a disorder in galactose metabolism.

Galactose utilization in *S. cerevisiae* occurs through the Leloir pathway (Fig. 1), in which galactose is converted to glucose-1-phosphate via galactose-1-phosphate. Glucose-1-phosphate is then converted to glucose-6-phosphate, which may enter both the Embden-Meyerhof-Parnas pathway and the pentose phosphate pathway. The genes encoding the enzymes of the Leloir pathway are tightly regulated; they are repressed by glucose and induced up to 1,000-fold by galactose (26). When galactose is absent from the medium, Gal80 inhibits the function of the transcriptional activator Gal4, which is required for *GAL* gene expression. Mig1 mediates glucose repression by repressing expression of *GAL1* and *GAL4* (14, 19, 29), while Gal6 also exerts negative control on the *GAL* system, although the exact mechanism remains to be elucidated (54). In a previous study we found that overexpression of *GAL4* and deletion of *GAL6*, *GAL80*, and *MIG1* resulted in galactose uptake rates that were increased 26% and 41%, respectively (33). These findings were believed to result from a general up-regulation of the complete Leloir pathway. In order to study this further and to identify possible global regulatory effects of these mutations, we used DNA arrays to analyze the reference strain and two mutants. In this analysis we were particularly interested in identifying novel targets for further improvement of the flux through the pathway. Analysis of different mutants with the objective of identifying targets for metabolic engineering and subsequently pursuing a target to improve the properties of a strain has been referred to as inverse metabolic engineering (4, 8). This study is an example of this approach, which may be very useful in all cases in which a pedigree of strains with different properties is available.

MATERIALS AND METHODS

Yeast strains. All *S. cerevisiae* strains used in this study were generated from the prototrophic CEN.PK113-7D reference strain (*MAT***a** *SUC2 MAL2-8*), which is a strain that is often used for physiological studies (49). A *GAL4*-overexpressing strain containing the 2μ high-copy-number vector pBM959 with *GAL4* behind its native promoter and *URA3* as a marker and a Δ*gal6* Δ*gal80* Δ*mig1* strain were constructed previously (33). A strain overexpressing *PGM2* was constructed

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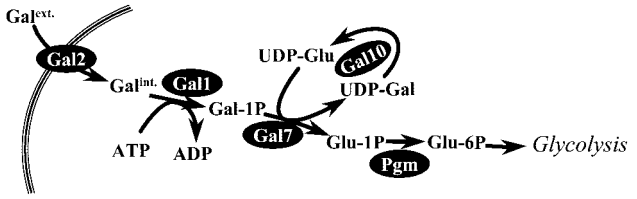

FIG. 1. Galactose utilization pathway in *S. cerevisiae*. Extracellular galactose (Gal^{ext}) is transported into the cell by galactose permease (Gal2) and then converted to glucose-6-phosphate (Glu-6P) by reactions catalyzed by galactokinase (Gal1), galactose-1-phosphate uridylyltransferase (Gal7), UDP-glucose 4-epimerase (Gal10), and phosphoglucomutase (Pgm) encoded by *PGM1* and *PGM2*.

by transforming CEN.PK113-5D (*MAT***a** *SUC2 MAL2-8 ura3-52*) with the 2μ high-copy-number vector p*PGM2* (25) containing *PGM2* behind the *PMA1* gene promoter and *URA3*.

Batch cultivation. Aerobic batch cultivation was carried out in well-controlled laboratory fermentors with a working volume of 2 or 4 liters. To obtain samples for analyses of RNA, biomass, and extracellular metabolites, a defined medium (51) was used, which contained (per liter) 15 g galactose, 5.0 g $(NH_4)_2SO_4$, 3.0 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, and trace metals and vitamins as described previously (51). Separate cultivations were performed to obtain samples for measurement of intracellular metabolites, and the same medium was used except that it contained twice the amounts of galactose, salts, and trace metals. To all cultures 50 μl/liter antifoam 289 (Sigma-Aldrich, St. Louis, MO) was added to avoid foaming. Galactose was autoclaved separately from the mineral medium and then added to the fermentor together with a sterile filtered solution containing the vitamins.

Precultures were inoculated from plate cultures, which were inoculated directly from frozen stocks, and were grown at 30°C on selective media. The cultures were grown at 30°C and 150 rpm in cotton-stoppered, 500-ml Erlenmeyer flasks with baffles containing 100 ml of a medium (pH 6.5) similar to the medium in the fermentors but containing different concentrations of galactose (20 g/liter), $(NH_4)_2SO_4$ (7.5 g/liter), and KH_2PO_4 (14 g/liter). Precultures in the exponential phase were used to inoculate the aerobic batch cultures at an initial concentration of 1 mg (dry weight)/liter. The aerobic batch cultures were incubated at 30°C with a stirrer speed of 800 rpm and with air added at a flow rate of 1 liter per liter of starting volume per min. The concentrations of dissolved oxygen in selected cultures were measured with a Mettler Toledo polarographic electrode and remained greater than 70%. The off-gas passed through a cooled condenser to avoid evaporation of ethanol from the bioreactors. The pH was kept at 5.0 by automatic addition of 4 M KOH.

Measurement of biomass and analysis of extracellular metabolites. The dry weight of biomass was determined as described previously (33). Culture samples used for determination of glucose, ethanol, glycerol, acetate, pyruvate, and succinate concentrations were filtered through a 0.45-μm cellulose acetate filter (Osmonics, Minnetonka, MN) immediately after sampling, and the filtrate was frozen at −20°C until further analysis. The concentrations of the metabolites were determined by high-pressure liquid chromatography as described previously (28).

Sampling and RNA isolation. Samples for RNA isolation were taken by rapidly transferring 20 ml of culture into a tube with 35 to 40 ml of crushed ice, which decreased the temperature to less than 2°C in less than 10 s. Cells were quickly pelleted by centrifugation (4,500 rpm at 0°C for 2 min), instantly frozen in liquid nitrogen, and then stored at −80°C. Total RNA was extracted by using a FastRNA kit, Red (Bio 101, Inc., Vista, CA) after the samples were thawed on ice.

Probe preparation and hybridization to arrays. mRNA extraction, cDNA synthesis, cRNA synthesis, and labeling, as well as hybridization to Affymetrix yeast genome S98 arrays (Affymetrix, Santa Clara, CA), were performed essentially as described in the Affymetrix users' manual (1). Washing and staining of arrays were performed using a GeneChip Fluidics Station 400, and the arrays were scanned with an Affymetrix GeneArray scanner.

Data acquisition and analysis. Acquisition and quantification of array images, as well as primary data analysis, were performed using the Affymetrix software package Microarray Suite, version 4.0.1. All arrays were globally scaled to a target value of 500 using the average signal from all gene features using Microarray Suite, version 4.0.1. The Affymetrix S98 yeast microarrays contain probe sets representing 9,335 distinct transcription features. After exclusion of all probe sets not assigned yORF abbreviations in the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/) and all probe sets representing groups of genes, which were already represented as singletons, 6,072 probe sets remained for analysis. Statistical analyses were performed by using an analysis of variance (ANOVA) for all probe sets representing genes identified as present by the Micrarray Suite software (version 4.0.1) in at least one array. The ANOVA calculated the probability (P) that several conditions all came from the same distribution. ($1 - P$) gave the probability that expression of a gene was significantly changed in at least one strain (21). A Bonferroni correction for multiple testing was used to adjust the significance level for transcripts whose abundance had changed. When k genes are tested, the critical significance level (α level) is divided by k returning a new cutoff value (α/k), which on a global scale gives α probability of having one false positive (5, 21). Fold changes were calculated by using the average expression levels of each triplicate.

Measurement of enzyme activities. Cell extracts were produced with the help of a Fastprep FP120 instrument (Savant Instruments, New York) as previously described (28). In vitro enzyme activities of phosphoglucomutase and galactose-1-phosphate uridyl transferase (Gal7) were assayed at 30°C by monitoring the NADPH production in a 1-ml reaction mixture at 340 nm using a spectrophotometer (HP 8353 UV-VIS system with Chemstation software from Hewlett-Packard, Palo Alto, CA) for at least two different concentrations of cell extract. The activity of phosphoglucomutase was measured in a reaction mixture containing 80 mM triethanolamine (pH 7.6), 1.7 mM $MgCl_2$, 20 μM glucose 1,6-diphosphate, 0.9 mM EDTA, 0.2 mM $NADP^+$, and 0.47 U/ml glucose-6-phosphate dehydrogenase, and the reaction was started by adding glucose-1-phosphate to a concentration of 3.5 mM as described previously (6, 13). Gal7 activities were determined as described previously (6, 13) with a reaction mixture consisting of 100 mM glycine-KOH (pH 8.7), 5 mM $MgCl_2$, 5 μM glucose-1,6-diphosphate, 5 mM dithiothreitol, 0.4 mM UDP-glucose, 0.8 mM $NADP^+$, 0.15 U/ml phosphoglucomutase, and 0.20 U/ml glucose-6-phosphate dehydrogenase, and the reaction was started by adding galactose-1-phosphate to a concentration of 0.8 mM. Galactokinase (Gal1) activity was measured by a method modified from the method described by Mizoguchi et al. (27). The reaction mixture contained 0.1 M phosphate buffer (pH 7.5), 3.0 mM $MgCl_2$, 1.8 mM NaF, 1.5 mM ATP, and four different concentrations of cell extract. After 5 min of incubation at 30°C, addition of 2.5 mM galactose started the reaction, and the reaction mixture was incubated for another 5 min and then boiled for 2 min. The amount of galactose consumed was determined by measuring the residual concentration of galactose enzymatically with a Boehringer Mannheim galactose kit (Boehringer Mannheim, Mannheim, Germany) after centrifugation (10 min, 1,000 × g). The protein contents of cell extracts were determined by the method of Lowry et al. (24), using essentially fatty acid-free (≥96%) bovine serum albumin (Sigma-Aldrich, St. Louis, MO) as the standard.

Analysis of intracellular metabolites. Cell extracts were obtained and clean-up of sugar phosphates (galactose-1-phosphate, galactose-6-phosphate, glucose-6-phosphate, fructose-6-phosphate) was performed by using the method described previously (45). Cell extracts were obtained by using cold methanol as the quenching agent and chloroform as the extraction solvent. In addition, clean-up of sugar phosphates was performed using solid-phase extraction (45). Sugar phosphate contents were determined by anion-exchange chromatography with pulsed amperometric detection as described by Smits et al. (45).

RESULTS

Genome-wide transcript analysis. Previously, we determined that overexpression of *GAL4* or deletion of the *GAL6*, *GAL80*, and *MIG1* genes resulted in an increased rate of uptake of galactose (33). Aiming to describe the global transcriptional effects of these modifications and to identify novel targets for further improvement of galactose uptake, we examined the transcript profiles of the two strains and the corresponding reference strain, strain CEN.PK113-7D. Cells were grown under well-controlled conditions in aerobic batch cultures on a minimal medium with an initial galactose concentration of 15 g/liter. A typical fermentation profile of the reference strain is shown in Fig. 2A. All the strains had similar specific growth rates (0.17 ± 0.01 h^{-1}), which were slightly higher than those reported previously (33). In the present study the biomass was analyzed directly, whereas in the previ-

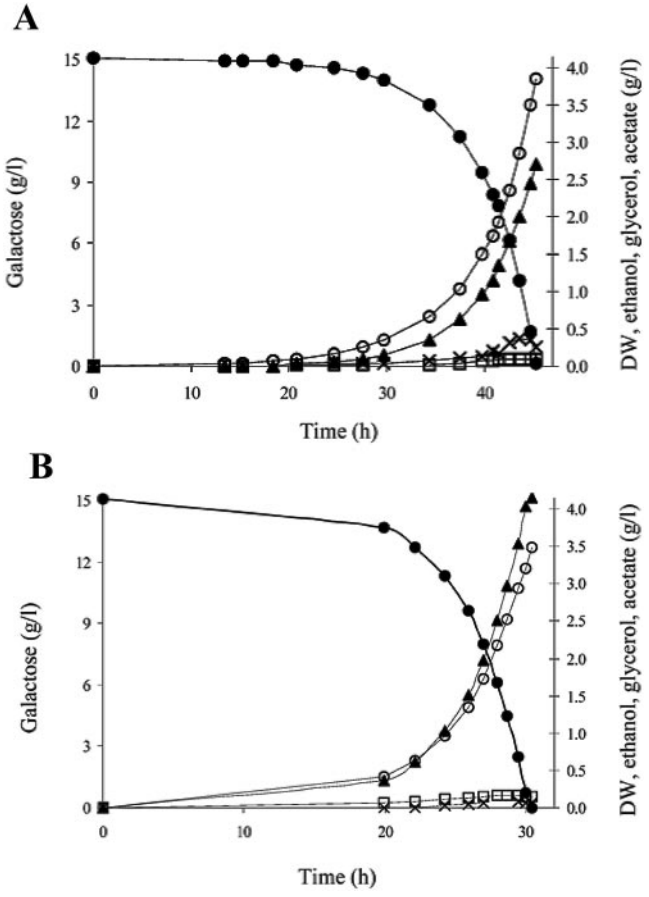

FIG. 2. Fermentation profiles of aerobic batch cultures grown on galactose. (A) Reference strain. (B) *PGM2* strain. The graphs show the concentrations of residual galactose (●), the cell dry weights (DW) (○), the concentrations of ethanol (▲), the concentrations of glycerol (×), and the concentrations of acetate (□) in the cultures at different times.

ous study (33) samples were frozen before analysis, and this may have affected the biomass concentration measured and thus the determination of the maximum specific growth rate. All strains were cultivated in triplicate, and one whole-genome transcription analysis was performed for each replicate by using cells harvested at a residual galactose concentration of 7.5 ± 2.0 g/liter in the middle of the exponential growth phase.

An ANOVA was performed for transcripts of the 5,848 open reading frames (ORFs) in *S. cerevisiae* that were detectable on at least one of the DNA arrays. This yielded only 25 ORFs that exhibited a significant change in the transcript levels in at least one of the two mutants when we used a global likelihood of getting one false positive (cutoff at $P = 1.71 \times 10^{-4}$). For most of these ORFs the transcript levels were changed only in the *GAL4*-overexpressing strain (called the *GAL4* strain) (Table 1 shows the 30 highest-scoring genes). The genome-wide analysis confirmed that the transcript levels of *MIG1*, *GAL4*, *GAL6*, and *GAL80* were altered in the directions that would be expected from the genetic modifications of the strains. However, the limited reduction in the *GAL80* transcript level in the Δ*gal6* Δ*gal80* Δ*mig1* strain was surprisingly small and unexpected as the coding region *GAL80* was almost completely deleted (33). As the *GAL80* transcript was down-regulated with high significance and we felt confident that *GAL80* was deleted, we assumed that the relatively high transcript level measured was some kind of background.

The *GAL6* transcript level was reduced in the Δ*gal6* Δ*gal80* Δ*mig1* strain, but it also showed possible twofold up-regulation in the *GAL4* strain. According to a Student's *t* test assuming unequal variances, this twofold up-regulation was significant with a 99.0% probability when we did not correct for multiple testing. In contrast to our expectations, *GAL6* was the only gene encoding significantly up-regulated transcripts with a Gal4 binding site in its promoter. The hypothetical ORF YIL057C also had a putative Gal4 binding site in its promoter (17), but the level of transcript was lower in the *GAL4* strain. Hence, most of the significantly changed transcript levels were likely to be the result of secondary effects of overexpression of *GAL4*. Gal4 is a member of the zinc cluster proteins, which is a subfamily of 54 transcriptional regulators in *S. cerevisiae* (2, 47), and it binds to a upstream activation sequence (UAS_{GAL}) that is characterized by the sequence 5′-CGG-N_{11}-CCG-3′ (16). Furthermore, it is able to bind a variety of sites having the general formula 5′-(A/C)GG-N_{10-12}-CCG-3′ (50). It has previously been reported that overexpression of *GAL4*, probably due to titration of other transcription factors, can inhibit transcription of several genes (15), and data have suggested that under appropriate circumstances Gal4 may be able to function in place of related family members to activate expression (11). Several of the genes encoding the significantly altered transcripts (*MDH2*, *MAL* genes, YGL157W, *URA1*, *HXT10*) are or might be regulated by zinc cluster proteins, while *ZAP1* and YDR520C are themselves zinc protein cluster members.

HXT10, which encodes a hexose transporter capable of transporting galactose (52), was up-regulated in both mutants, which could explain the increased galactose uptake in the two mutants. However, as *HXT10* was expressed at a low level compared to the level of the main galactose transporter gene, *GAL2*, and since overexpression of *GAL2* results in a decreased galactose uptake rate (32), we decided not to pursue this possible target for further increasing galactose utilization. The transcript level of the hypothetical ORF YKL031W was also higher in both mutants. This ORF is predicted to encode a 137-amino-acid protein which binds phosphoinositol 4,5-biphosphate in vitro (55), and it contains two predicted transmembrane segments (39); however, it has no obvious role in carbon metabolism, and therefore, we did not pursue this possible target.

Analysis of gene subset. The genome-wide transcript analysis of the two mutants did not indicate any clear reason for the mutants' increased galactose uptake rates. Therefore, we focused on the transcription results for the *GAL* genes, which are the most obvious candidates for key enhancers. Besides *GAL4*, *GAL6*, and *GAL80*, only the *PGM2* transcript, encoding the major isoform of phosphoglucomutase, had a high probability of being changed (Table 2). Taking multiple testing of the 10 *GAL* genes into consideration, there was a 94% probability of a changed *PGM2* transcript level in at least one of the two mutant strains. The *PGM2* transcript level increased 1.7- and 1.3-fold in the *GAL4* strain and the Δ*gal6* Δ*gal80* Δ*mig1* mutant, respectively. In order to verify that the concentration of active phosphoglucomutase indeed increased and that the con-

TABLE 1. The thirty genes with the highest probability of changed transcript levels in at least one of the strains according to an ANOVA test

ORF	Gene	Function	Fold change[a]		P[b]
			GAL4 strain	Δ*gal6* Δ*gal80* Δ*mig1* strain	
YGL157W		Unknown	3.7	−1.5	3.53×10^{-8}
YEL021W	*URA3*	Pyrimidine biosynthesis	5.2	−1.2	5.66×10^{-7}
YPL248C	*GAL4*	Galactose regulation	23	1.3	5.95×10^{-7}
YBR298C	*MAL31*	Maltose metabolism	−1.4	1.1	3.85×10^{-6}
YML051W	*GAL80*	Galactose regulation	1.3	−2.1	7.90×10^{-6}
YJL056C	*ZAP1*	Transcriptional regulation	2.3	1.1	1.31×10^{-5}
YLR035C	*MLH2*	DNA repair	3.1	−1.5	2.60×10^{-5}
YBR093C	*PHO5*	Phosphate metabolism	32	1.0	3.11×10^{-5}
YIL057C		Unknown	−1.2	1.8	3.77×10^{-5}
YGL130W	*CEG1*	mRNA capping	2.1	1.0	5.54×10^{-5}
YOL143C	*RIB4*	Riboflavin biosynthesis	1.7	1.2	5.76×10^{-5}
YKL031W		Unknown	9.4	1.9	6.04×10^{-5}
YLR286C	*CTS1*	Cell wall biogenesis	−1.4	1.1	7.30×10^{-5}
YNL239W	*GAL6*	Galactose regulation	2.0	<−100	7.65×10^{-5}
YGR022C		Unknown	10	1.0	9.68×10^{-5}
YEL069C	*HXT13*				
YDL245C	*HXT15*				
YJR158W	*HXT16*				
YNR072W	*HXT17*	Hexose transporters	1.0[c]	3.2[c]	9.95×10^{-5}
YBR299W	*MAL32*				
YGR292W	*MAL12*	Maltose metabolism	−2.3[c]	1.2[c]	1.07×10^{-4}
YOL126C	*MDH2*	Gluconeogenesis	2.1	−1.2	1.13×10^{-4}
YFL011W	*HXT10*	Hexose transporter	2.7	1.7	1.19×10^{-4}
YMR305C	*SCW10*	Mating	−1.6	−1.2	1.37×10^{-4}
YKL216W	*URA1*	Pyrimidine biosynthesis	1.7	1.1	1.42×10^{-4}
YDR520C		Unknown	1.7	1.1	1.42×10^{-4}
YGL035C	*MIG1*	Glucose repression	−1.3	−17	1.46×10^{-4}
YPL187W	*MFα1*	Mating	−2.1	−2.1	1.48×10^{-4}
YMR199W	*CLN1*	Cell cycle	−1.7	−1.1	1.63×10^{-4}
YJR159W	*SOR1*	Fructose metabolism			
YDL246C		Unknown	2.2[c]	1.4[c]	1.83×10^{-4}
YMR176W	*ECM5*	Cell wall biogenesis	2.1	1.1	2.04×10^{-4}
YOL058W	*ARG1*	Arginine biosynthesis	1.2	1.0	2.19×10^{-4}
YOR378W		Unknown	5.8	1.5	2.49×10^{-4}
YGR249W	*MGA1*	Filamentous growth	−3.2	−2.4	2.53×10^{-4}

[a] Fold change compared to the reference strain.

[b] Probability of unchanged transcript levels in all three strains according to an ANOVA test. The lower the value, the higher the probability of significantly changed expression. When a global likelihood of getting one false positive was used, the cutoff was at $P = 1.71 \times 10^{-4}$, calculated by using a Bonferroni correction for multiple testing.

[c] The probe set on DNA arrays represents more than one ORF.

centrations of other Gal enzymes did not increase, in vitro enzyme activities of Gal1, Gal7, and phosphoglucomutase from the same cultures were measured. The results supported the conclusion drawn from the transcript analysis (Table 2); i.e., only phosphoglucomutase activity (encoded by both *PGM1* and *PGM2*) was consistently increased in the two mutants. As Pgm2 was the isoform assumed to have higher activities in the mutants, we could use Kacser's theory of large deviations (44) to calculate the flux control coefficient for phosphoglucomutase. The flux control coefficient described the relative contribution of the enzyme to the control of flux in the pathway, using the data from the two mutants. We found that this enzyme has a flux control coefficient of 0.47 to 0.87 in the reference strain (0.47 was found by using the data for the *GAL4* strain, and 0.87 was found by using the data for the Δ*gal6* Δ*gal80* Δ*mig1* strain). Even though these calculations are relatively uncertain, they indicate that phosphoglucomutase activity exerted substantial control on the flux through the Leloir pathway in the reference strain.

Overexpression of *PGM2*. On the basis of the transcript analysis, *PGM2* was found to be up-regulated in the mutants with a higher flux through the Leloir pathway. To evaluate whether Pgm2 could be a target for improving the flux through the galactose utilization pathway, a strain overexpressing *PGM2* (called the *PGM2* strain) was constructed. This strain was cultivated in triplicate under the same conditions that were used for the other strains. The *PGM2* strain had a significantly increased maximum specific galactose uptake rate compared to the rates for both the reference strain and the two other mutants (Fig. 3). Overexpression of *PGM2* resulted in a 70% increase in the maximum specific galactose uptake rate and a threefold-higher maximum specific ethanol production rate compared to the rates for the reference strain. Detailed physiological data for all the strains analyzed are shown in Table 3, and a fermentation profile for the *PGM2* strain is shown in Fig. 2B. The maximum specific growth rate on galactose of the *PGM2* strain was $0.23 \pm 0.02\ h^{-1}$, compared to $0.17 \pm 0.01\ h^{-1}$ for the reference strain. Due to the increase in both the specific

TABLE 2. Transcript analysis of the *GAL* genes and analysis of in vitro enzyme activities

Gene	Transcription			In vitro enzyme activity			
	Fold change[a]		P^b	Activity in reference strain (U/mg protein)[c]	Fold change[a]		P^b
	GAL4 strain	Δ*gal6* Δ*gal80* Δ*mig1* strain			*GAL4* strain	Δ*gal6* Δ*gal80* Δ*mig1* strain	
GAL4	23	1.3	6.0×10^{-7}				
GAL80	1.3	−2.1	7.9×10^{-6}				
GAL6	2.0	<−100	7.7×10^{-5}				
PGM2	1.7	1.3	6.1×10^{-3}	0.14 ± 0.02[d]	2.6	1.5	3.2×10^{-3}
GAL7	1.0	1.1	0.17	0.33 ± 0.13	1.6	−1.3	2.4×10^{-2}
GAL3	1.7	1.0	0.18				
GAL2	1.0	1.1	0.38	ND[e]			
GAL10	1.0	1.1	0.46	ND			
PGM1	1.2	1.1	0.57	0.14 ± 0.02[d]	2.6	1.5	3.2×10^{-3}
GAL1	1.0	1.0	0.99	1.04 ± 0.24	−1.2	1.2	0.43

[a] Fold change compared to the reference strain.
[b] Probability of unchanged transcript levels in all three strains according to an ANOVA test.
[c] The specific enzyme activities are expressed as U/mg protein (μmol/min/mg protein) and are averages ± standard deviations for determinations for three independent reference batch cultures.
[d] Total phosphoglucomutase activity.
[e] ND, not determined.

galactose uptake rate and the specific growth rate, the volumetric galactose uptake was much greater in the *PGM2* strain, and the exponential growth phase was 30% shorter than that of the reference strain. In vitro enzyme activity measurements verified that the concentration of active phosphoglucomutase indeed increased in the *PGM2* strain, which exhibited 17-fold-higher activity. However, the in vitro enzyme activity of Gal7 also increased approximately threefold in this strain, while the activity of Gal1 was unchanged.

Intracellular hexose-6-phosphates. To clarify the mechanisms which cause the effects of overexpression of *PGM2*, we measured the intracellular levels of glucose-6-phosphate, galactose-1-phosphate, galactose-6-phosphate, and fructose-6-phosphate. In previous studies we consistently found that galactose-6-phosphate was present in yeast cells grown on galactose (unpublished data), and we assume that this metabolite is formed by the action of phosphoglucomutase on galactose-1-phosphate, as phosphoglucomutase previously has been reported to catalyze this reaction in vitro, although at a low rate (23, 38). Cultivation with twice the amounts of galactose, salts, and trace metals compared to the previous cultivation was performed in order to obtain a higher concentration of biomass, and samples were collected from the exponential growth phase to measure the levels of three intracellular metabolites downstream of phosphoglucomutase in the metabolism. The results showed that there were increased intracellular concentrations of glucose-6-phosphate, fructose-6-phosphate, and galactose-6-phosphate in the strain overexpressing *PGM2*, while the data for galactose-1-phosphate were inconclusive due to high standard deviations for some of the strains (Table 4). This clearly indicates that there was increased phosphoglucomutase activity, and the increased levels of glucose-6-phosphate and fructose-6-phosphate may play an important role in ensuring a higher glycolytic flux in the *PGM2* strain.

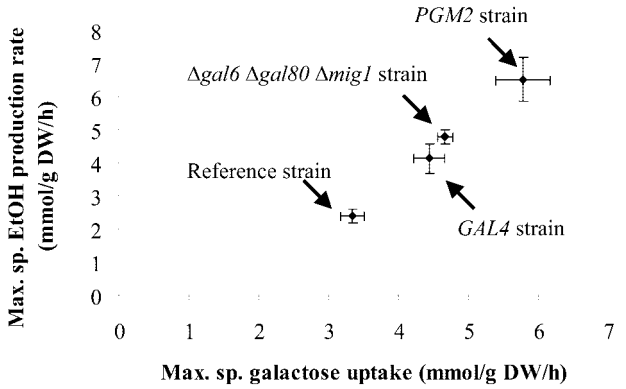

FIG. 3. Maximum specific ethanol production rates and maximum specific galactose uptake rates determined in aerobic batch cultures grown on galactose for the reference strain, the *GAL4* strain, the Δ*gal6* Δ*gal80* Δ*mig1* strain, and the *PGM2* strain. Each point is the average of three replicates, and the error bars indicate standard deviations for the replicates. DW, dry weight; EtOH, ethanol.

DISCUSSION

In the current study we found that overexpression of phosphoglucomutase results in increased flux through the Leloir pathway. This finding is surprising as this enzyme has generally not been considered to be an enzyme that exerts any control over the flux through the galactose utilization pathway, since the basal level of expression of phosphoglucomutase is quite high and expression increased only three- to fourfold in response to the presence of galactose (31). Furthermore, phosphoglucomutase has high affinities for its substrates (12), which often indicates that an enzyme has low flux control. Moreover, increased expression of only one enzyme-encoding gene is often insufficient to increase the flux of an entire pathway that the enzyme is part of, as illustrated by the efforts to increase the flux through glycolysis (41, 46). The positive effect of overexpression of *PGM2* found here, however, may well be due to a combination of a direct effect of a higher Pgm2 activity and secondary effects, such as increased activity of Gal7 or a decreased level of a toxic intermediate. The slow growth on galactose of the reference strain indicates a limitation or the

TABLE 3. Key physiological data for the strains evaluated in this study during exponential growth

Strain	Maximum specific galactose uptake rate (mmol/g [dry wt]/h)[a]	Maximum specific growth rate (h^{-1})	Biomass yield (g [dry wt]/g Gal)[b]	Ethanol yield (mmol/g Gal)[b]	Maximum specific ethanol production rate (mmol/g [dry wt]/h)[c]
Reference	3.33 ± 0.17[d]	0.17 ± 0.01	0.28 ± 0.02	3.91 ± 0.22	2.39 ± 0.22
GAL4	4.44 ± 0.22	0.18 ± 0.01	0.22 ± 0.01	5.21 ± 0.65	4.13 ± 0.43
Δ*gal6* Δ*gal80* Δ*mig1*	4.67 ± 0.11	0.18 ± 0.00	0.22 ± 0.01	5.64 ± 0.22	4.78 ± 0.22
PGM2	5.78 ± 0.39	0.23 ± 0.02	0.22 ± 0.01	6.30 ± 0.22	6.51 ± 0.65

[a] The maximum specific galactose uptake rate was calculated from the biomass yield and the maximum specific growth rate.
[b] Obtained from the slope of the linear curve when the biomass or metabolite concentration was plotted versus the galactose concentration during exponential growth.
[c] The maximum specific ethanol production rate was calculated from the ethanol yield and the maximum specific galactose uptake rate.
[d] The values are averages ± standard deviations for three independent cultures.

presence of a toxic intermediate, such as galactose-1-phosphate, an effect that may be reduced in the *PGM2* strain, as Gal7 and possibly also phosphoglucomutase use galactose-1-phosphate as a substrate.

PGM2 is induced by different stress conditions via the four stress response elements present in its promoter (10, 48), illustrating its dual role in metabolism. Its many functions in galactose metabolism, synthesis of UDP-glucose and glycogen, and trehalose metabolism explain the high basal level of expression of *PGM2* in the absence of galactose. However, *PGM2* also contains one putative Gal4-binding site in its promoter (17), suggesting that *PGM2* is transcriptionally regulated by Gal4, resulting in the three- to fivefold up-regulation of *PGM2* upon galactose induction (31). Therefore, the higher levels of *PGM2* transcript identified in the *GAL4* strain and the Δ*gal6* Δ*gal80* Δ*mig1* strain were not in themselves surprising, but what was surprising were the unchanged transcript levels of *GAL1*, *GAL2*, *GAL7*, and *GAL10*. Apparently, these genes were expressed at their optimal levels in the reference strain, while *PGM2* still had room for increased expression, presumably due to its lesser dependence on Gal4 for its regulation. The transcript level of *PGM2* and in vitro phosphoglucomutase activity were higher in the strain with *GAL4* overexpression than in the Δ*gal6* Δ*gal80* Δ*mig1* strain, whereas the specific galactose uptake rate was higher in the Δ*gal6* Δ*gal80* Δ*mig1* strain. This shows that increased Pgm2 activity is not the sole cause for increased galactose uptake rates; other factors must be important as well. One such candidate is Gal6, which affects galactose metabolism negatively (33). The transcript level of *GAL6* increased twofold in the strain with *GAL4* overexpression, and this might have had an unidentified negative effect that counteracted the higher transcript level of *PGM2* compared to that in the Δ*gal6* Δ*gal80* Δ*mig1* strain. Deletion of *GAL6* alone results in a 24% increase in the galactose uptake rate (33) and has also been reported to result in increased amounts of *GAL1*, *GAL2*, and *GAL7* mRNA (54). In agreement with the previously observation of Ideker and coworkers (17), we did not observe any effect on the amounts of *GAL1*, *GAL2*, or *GAL7* mRNA due to deletion of *GAL6* during growth on galactose. This may indicate that Gal6 has a function different from an effect on the concentration of *GAL* gene mRNA (e.g., it may affect translation).

Overexpression of *PGM2* resulted in increased conversion of glucose-1-phosphate to glucose-6-phosphate, thereby enabling more efficient shunting of sugar from the Leloir pathway into glycolysis. This may be seen in the higher intracellular concentrations of glucose-6-phosphate, fructose-6-phosphate, and maybe also galactose-6-phosphate in the strain overexpressing *PGM2*. The increased intracellular levels of glucose-6-phosphate and fructose-6-phosphate may ensure that the flux through glycolysis could be increased, and this may have a positive effect on galactose utilization through more efficient regeneration of ATP required for phosphorylation of galactose. Galactose-6-phosphate can be formed from galactose-1-phosphate in a reaction catalyzed by phosphoglucomutase (23, 38). Thus, the increased level of galactose-6-phosphate in the *PGM2*-overexpressing strain may also reflect increased activity of Pgm2.

Besides providing further insight into galactose metabolism, the present study is an example of how genome-wide transcript analysis can be used in an inverse metabolic engineering strategy. DNA arrays are very efficient for genome-wide screening of variations in transcript levels, but as illustrated here, changes in fluxes may be associated with very small changes in gene transcripts, and it is inherently difficult to identify genes that have small changes in transcription. Thus, even for carefully replicated cultures more false positives are observed with changes of less than twofold than with greater changes when a statistical analysis is performed (37). Changes less than twofold are often not considered significant in transcript analyses (7,

TABLE 4. Intracellular concentrations of hexose phosphates during exponential growth

Strain	Galactose-1-phosphate concn (μmol/g [dry wt])	Glucose-6-phosphate concn (μmol/g [dry wt])	Galactose-6-phosphate concn (μmol/g [dry wt])	Fructose-6-phosphate concn (μmol/g [dry wt])
Reference	0.43 ± 0.13[a]	0.36 ± 0.09	0.08 ± 0.03	0.16 ± 0.07
GAL4	0.28 ± 0.04	0.32 ± 0.10	0.06 ± 0.01	0.13 ± 0.04
Δ*gal6* Δ*gal80* Δ*mig1*	0.30 ± 0.07	0.39 ± 0.01	0.08 ± 0.03	0.16 ± 0.02
PGM2	0.47 ± 0.22	0.75 ± 0.28	0.27 ± 0.06	0.53 ± 0.18

[a] For the reference strain the data are the averages ± standard deviations for nine values obtained at three different times during exponential growth from each of two separate cultures. For the remaining strains the data are the averages ± standard deviations for at least three values obtained collected at three different times from one culture.

18, 22, 37) as a result of the increasing false discovery rate with decreasing fold changes. Some approaches to overcome this problem are increasing the number of replicates and applying the strategy described here of zooming in on specific genes and also analyzing several strains with different degrees of the desired phenotype, thereby increasing the statistical confidence in possible gene targets with low, but significant, fold changes. This study shows that it is advantageous to analyze two or more strains with improved properties in order to obtain a list of candidate genes for which there are similar trends in the transcript profiles. However, the transcript level of *PGM2* did not appear to be significantly changed from the genome-wide analysis, and it changed only when a relevant subset of genes was tested. Increasing the number of replicates would have increased the likelihood of finding *PGM2* in a whole-genome test as the number of false negatives drops substantially with more replicates (21), but with the approach of analyzing a gene subset more replicates were unnecessary. A key factor for success of this strategy is, however, the correct choice of genes in the analysis. The genes may be chosen either based on physiological knowledge, though the use of metabolic information, as demonstrated recently (9), or through the use of model-guided analysis of transcript data (36).

ACKNOWLEDGMENTS

We thank Lene Christiansen for her help with transcript analysis and Wian de Jongh for his work on measuring enzyme activities and intracellular metabolites. Plasmid p*PGM2* was a kind gift from Monica Montero-Lomeli, Universidade Federal Do Rio Janeiro, Brazil.

This work was financed in part by the Danish Biotechnology Instrument Center (DABIC).

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