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Molecular Analysis of a *Saccharomyces cerevisiae* Mutant with Improved Ability To Utilize Xylose Shows Enhanced Expression of Proteins Involved in Transport, Initial Xylose Metabolism, and the Pentose Phosphate Pathway

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Differences between the recombinant xylose-utilizing *Saccharomyces cerevisiae* strain TMB 3399 and the mutant strain TMB 3400, derived from TMB 3399 and displaying improved ability to utilize xylose, were investigated by using genome-wide expression analysis, physiological characterization, and biochemical assays. Samples for analysis were withdrawn from chemostat cultures. The characteristics of *S. cerevisiae* TMB 3399 and TMB 3400 grown on glucose and on a mixture of glucose and xylose, as well as of *S. cerevisiae* TMB 3400 grown on only xylose, were investigated. The strains were cultivated under chemostat conditions at a dilution rate of 0.1 h^{-1}, with feeds consisting of a defined mineral medium supplemented with 10 g of glucose liter^{-1}, 10 g of glucose plus 10 g of xylose liter^{-1} or, for *S. cerevisiae* TMB 3400, 20 g of xylose liter^{-1}. *S. cerevisiae* TMB 3400 consumed 31% more xylose of a feed containing both glucose and xylose than *S. cerevisiae* TMB 3399. The biomass yields for *S. cerevisiae* TMB 3400 were 0.46 g of biomass g of consumed carbohydrate^{-1} on glucose and 0.43 g of biomass g of consumed carbohydrate^{-1} on xylose. A K_s value of 33 mM for xylose was obtained for *S. cerevisiae* TMB 3400. In general, the percentage error was <20% between duplicate microarray experiments originating from independent fermentation experiments. Microarray analysis showed higher expression in *S. cerevisiae* TMB 3400 than in *S. cerevisiae* TMB 3399 for (i) *HXT5*, encoding a hexose transporter; (ii) *XKS1*, encoding xylulokinase, an enzyme involved in one of the initial steps of xylose utilization; and (iii) *SOL3*, *GND1*, *TAL1*, and *TKL1*, encoding enzymes in the pentose phosphate pathway. In addition, the transcriptional regulators encoded by YCR020C, YBR083W, and YPR199C were expressed differently in the two strains. Xylose utilization was, however, not affected in strains in which YCR020C was overexpressed or deleted. The higher expression of *XKS1* in *S. cerevisiae* TMB 3400 than in TMB 3399 correlated with higher specific xylulokinase activity in the cell extracts. The specific activity of xylose reductase and xylitol dehydrogenase was also higher for *S. cerevisiae* TMB 3400 than for TMB 3399, both on glucose and on the mixture of glucose and xylose.

Xylose is one of the most abundant building blocks in hemicellulose (22) and thus represents a large fraction of all organic carbon. *Saccharomyces cerevisiae*, the preferred organism for industrial ethanol production, cannot utilize xylose. However, this trait is necessary to make the production of fuel ethanol from xylose-rich lignocellulose cost-effective (28).

Recently, a diploid strain of *S. cerevisiae* (26) was transformed with the genes coding for xylose reductase (XR) and xylitol dehydrogenase (XDH) from the xylose-utilizing yeast *Pichia stipitis*, as well as with the *S. cerevisiae* gene encoding xylulokinase (XK) (29a). The resulting transformant, *S. cerevisiae* TMB 3399, had a maximum specific growth rate of 0.025 h^{-1} when cultivated in a defined mineral medium supplemented with xylose. Chemical mutagenesis using ethyl methanesulfonate generated mutants with improved growth rate and xylose utilization. The best mutant selected was designated *S. cerevisiae* TMB 3400 and showed a >5-fold increase in growth rate, 0.14 h^{-1}, when cultivated under the same conditions as *S. cerevisiae* TMB 3399. The mutant, *S. cerevisiae* TMB 3400, also showed lower xylitol production than *S. cerevisiae* TMB 3399 when the strains were cultivated on xylose under oxygen-limited and anaerobic conditions.

Microarray technology permits the quantification of genome-wide mRNA expression (31). The technique offers the possibility to characterize differences in transcription level as a function of strain difference or cultivation condition. In *S. cerevisiae*, microarray technology has, among other things, been used to compare the mRNA expression during growth under aerobic and anaerobic conditions (25) and during a shift from growth on a fermentable carbon source to growth on a nonfermentable carbon source (10).

To identify genes beneficial for growth on xylose, microarray technology was used to compare mRNA expression in *S. cerevisiae* TMB 3399 and TMB 3400, as well as the growth of these two strains on glucose and xylose. *S. cerevisiae* TMB 3399 was cultivated in batch and chemostat cultures at a dilution rate of 0.1 h^{-1} in a defined mineral medium supplemented with either glucose or a mixture of glucose and xylose. *S. cerevisiae* TMB 3400 was cultivated under the same conditions, as well as by

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TABLE 1. Feed concentrations, residual substrate concentrations, and specific productivities from chemostat cultivation of *S. cerevisiae* strains TMB 3399 and TMB 3400 as determined with glucose and xylose as the carbon sources

S. cerevisiae strain	Avg ± SD[a]											C balance[b] (%)
	Feed concn ($g\ liter^{-1}$)		Residual concn ($g\ liter^{-1}$)		Biomass ($g\ liter^{-1}$)	Biomass yield (g of biomass g of consumed $carbohydrate^{-1}$)	Specific productivity (mg/g of biomass h^{-1})					
	Glucose	Xylose	Glucose	Xylose			$q_{glucose}$	q_{xylose}	$q_{xylitol}$	q_{CO_2}		
TMB 3399	10.8 ± 0.2		ND		5.1 ± 0.2	0.48 ± 0.01	−247 ± 3			143 ± 7		96
TMB 3400	10.8 ± 0.2		ND		4.9 ± 0.2	0.46 ± 0.01	−231 ± 6			136 ± 1		94
TMB 3399	10.2 ± 0.1	10.0 ± 0.2	0.09 ± 0.06	4.6 ± 0.1	7.4 ± 0.1	0.48 ± 0.01	−160 ± 1	−85 ± 2	2.5 ± 0.5	150 ± 1		101
TMB 3400	10.2 ± 0.1	10.0 ± 0.2	ND	2.9 ± 0.4	8.1 ± 0.4	0.47 ± 0.01	−142 ± 3	−97 ± 5	ND	155 ± 3		103
TMB 3400		20.8 ± 0.1		8.5 ± 0.12	5.4 ± 0.1	0.43 ± 0.01		−254 ± 2	1 ± 0.1	163 ± 1		94

[a] Values are averages of two independent cultivation experiments. ND, not detected.
[b] The generalized biomass formula $CH_{1.8}O_{0.5}N_{0.2}$ was used to calculate the carbon balances.

using a feed containing only xylose. At steady state, samples were withdrawn for microarray analysis; analysis of XR, XDH, and XK activities; and analysis of substrate consumption and product formation.

MATERIALS AND METHODS

Strains, medium, and cultivation conditions. The genes encoding XR and XDH from *P. stipitis*, as well as XK from *S. cerevisiae*, were integrated into the genome of *S. cerevisiae* TMB 3399 by using the plasmid YIpXR/XDH/XK (29a). YIpXR/XDH/XK carries the genes encoding XR, XDH, and XK, as well as the β-lactamase gene and a *HIS3* marker gene (6). In this plasmid, the gene encoding XR is under the control of the *ADH1* promoter, whereas the genes encoding XDH and XK are under the control of the *PGK1* promoter.

S. cerevisiae TMB 3399 and *S. cerevisiae* TMB 3400 were stored at −80°C and streaked on yeast extract-peptone-dextrose plates 1 day prior to the inoculation of the precultures. A defined mineral medium, including vitamins and trace elements, was used in all fermentation experiments (27). The carbon and energy source consisted of either 10 g of glucose, 10 g of glucose plus 10 g of xylose, or 20 g of xylose $liter^{-1}$. Antifoam (Dow Corning Antifoam RD Emulsion; BDH Laboratory Supplies, Poole, England) was added at a concentration of 0.5 ml per liter.

The precultures consisted of 100 ml of medium containing 10 g of glucose $liter^{-1}$ in 1,000-ml baffled shake flasks. They were incubated at 30°C and 140 rpm in an orbital incubator (INR-200; Gallenkamp, Leicester, United Kingdom). The precultures used for the cultivation of *S. cerevisiae* TMB 3400 on pure xylose were cultivated under the same conditions but with 20 g of xylose $liter^{-1}$ as the carbon source. The bioreactors were inoculated with 20 mg of cells harvested in the late exponential phase $liter^{-1}$.

Batch and chemostat fermentation was conducted at 30°C in 2-liter Biostat A bioreactors (B. Braun Biotech International, Melsungen, Germany). The working volume of the bioreactors was 1,200 ml, and 3 M NaOH was used to automatically maintain the pH at 5.5. The airflow rate of 1 liter min^{-1} was controlled by mass flow meters (HI-TECH; Bronkhorst, Ruurlo, The Netherlands), and the dissolved oxygen tension was at least 30% of the maximum. Duplicate fermentation experiments were performed for each feed composition.

Sampling, preparation of cRNA, microarray analysis, and analysis of substrates and products. Steady-state conditions were assumed when the carbon dioxide evolution, oxygen consumption, and OD_{620} had remained constant for three consecutive volume changes. This occurred at least six volume changes after feed initialization or feed change. Samples were then withdrawn for microarray analysis (2 × 50 ml), enzymatic assays (50 ml), and for the analysis of dry weight, substrate consumption and product formation (50 ml). The biomass concentrations were between 5 and 8 mg ml^{-1} (Table 1).

Cells for microarray analysis were kept on ice for 30 min. The cell samples were subsequently centrifuged for 5 min at 5,000 × *g* and 4°C. The cells were then washed twice with ice-cold AE buffer (50 mM sodium acetate, pH 5.2; 10 mM EDTA). The cell pellets were frozen in liquid nitrogen and stored at −80°C while awaiting further processing. Total RNA was isolated by using the hot-phenol method (21) and mRNA was purified by using the Oligotex mRNA Mini Kit (Qiagen, Hilden, Germany). The synthesis of cDNA was performed by using the Superscript choice system (Gibco-BRL/Life Technologies, Gaithersburg, Md.) and a T7-(dT)24 oligonucleotide primer (Genset, Paris, France). In vitro transcription was carried out by using the Enzo BioArray High-Yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, N.Y.). The cRNA was fragmented, and the hybridization mixture was prepared according to the recommendations of the manufacturer of the microarrays (Affymetrix, Santa Clara, Calif.). Hybridization, washing, staining, and scanning of the microarrays (GeneChip yeast genome S98 arrays) were performed by using a Hybridization Oven 320, a Fluidics Station 400, and a GeneArray scanner (all from Affymetrix). Data were collected and processed by using the Microarray Suite software, version 4.0 (Affymetrix).

Cells for enzymatic activity measurements were washed once with distilled water and treated with yeast protein extraction solution (Y-PER; Pierce, Rockford, Ill.) for 20 min at room temperature. The mixture was centrifuged for 5 min at 4°C and 5,000 rpm, and the supernatant was used for enzymatic assays. XR, XDH, and XK were assayed as previously described (6), but triethanolamine buffer at pH 7.0 was used for the XDH assay instead of glycine buffer at pH 9.0, since the high pH of the glycine buffer caused precipitation of components in the extraction solution.

Glucose, xylose, xylitol, succinate, glycerol, acetate, and ethanol were analyzed by high-pressure liquid chromatography, as has been described previously (29). The composition of the outgoing gas was monitored continuously with a carbon dioxide and oxygen monitor type 1308 (Brüel & Kjaer, Copenhagen, Denmark) (4) by using photoacoustic and magnetoacoustic detection for CO_2 and O_2, respectively. The cell dry weight was determined by filtering a known volume of the culture broth through a 0.45-μm (pore-size) Supor membrane (Gelman Sciences, Ann Arbor, Mich.). The filter was weighed after being washed with 3 volumes of double-distilled water and dried in a microwave oven for 15 min at 350 W. The cell dry weight was determined in triplicate.

Construction of strains with open reading frame YCR020c overexpressed and deleted. *E. coli* DH5α (Life Technologies, Rockville, Md.) was used for the cloning procedures. The p426ADH (ATCC 87377) multicopy vector (14) was used to overexpress the open reading frame YCR020c. This vector contains the β-lactamase gene, the *URA3* selection marker, and an *ADH1* promoter and *CYC1* terminator. The integrative plasmid YIpXR/XDH/XK (6) was used to introduce the genes encoding XR, XDH, and XK. The strains *S. cerevisiae* Y00000 (BY4741; *MAT***a** *his3-Δ1 leu2Δ0 met15Δ0*) and *S. cerevisiae* Y03500 (BY4741; *MAT***a** *his3-Δ1 leu2Δ0 met15Δ0 YCR020c::kanMX4*) were obtained from Euroscarf (Frankfurt, Germany).

Construction of xylose-utilizing strains with overexpression and deletion of YCR020c. All enzymes used for cloning and restriction analysis were obtained from Fermentas (Vilnius, Lithuania) unless stated otherwise. Standard techniques were used for cloning, transformation, and analysis (1). Chromosomal DNA from *S. cerevisiae* TMB 3400 was prepared by using the Easy-DNA kit (Invitrogen, Carlsbad, Calif.) and was used as the template for PCR amplification of the open reading frame YCR020C. The PCR product was obtained by using the upstream primer 5′-GCA CTA GT**A TG**A GCT GTA CCA CTG ATA AGT TA-3′ in combination with the downstream primer 5′-GCG AAT TCT TAG GCG TTG TAA CAA GAT TCA AAA-3′. The start codon is indicated in boldface, and the restriction sites for *BcuI* (*Spe*I) (upstream primer) and *Eco*RI (downstream primer) are underlined. The concentrations of nucleotides, *Pwo* DNA polymerase, primers, and Mg^{2+} ions were as recommended by the supplier (Roche, Mannheim, Germany). The PCR was performed in a GeneAmp PCR system 9700 (Perkin-Elmer Corp., Norwalk, Conn.), and the conditions employed were as follows: 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min (10 cycles); 94°C for 1 min, 61°C for 30 s, and 72°C for 1.5 min (15 cycles); and 94°C for 1 min, 61°C for 30 s, and 72°C for 2.5 min (10 cycles). The PCR product was

purified with the QiaQuick kit (Qiagen, Hilden, Germany) and, together with the vector p426 ADH, it was cleaved with *Bcu*I and *Eco*RI and ligated with T4 DNA ligase to form the plasmid pFW5.

S. cerevisiae TMB 3151 was created by integrating the YIpXR/XDH/XK plasmid (6), linearized by digestion with *Pst*I, into the *HIS3* locus of the laboratory strain *S. cerevisiae* CEN.PK 113-11C (*MAT***a** *his3-Δ1 ura3-52*). *S. cerevisiae* TMB 3151 was transformed with the plasmids p426ADH and pFW5, resulting in *S. cerevisiae* strains TMB 3152 and TMB 3155, respectively.

S. cerevisiae strains Y00000 and Y03500 were transformed with the YIpXR/XDH/XK plasmid in the same way as *S. cerevisiae* CEN.PK 113-11C and were named *S. cerevisiae* strains TMB 3159 and TMB 3160, respectively.

The recombinant strains, *S. cerevisiae* TMB 3152, TMB 3155, TMB 3159, and TMB 3160, were cultivated by using the same medium as described above. One-liter shake flasks containing 100 ml of medium supplemented with 20 g of glucose or 10 g of ethanol liter^{-1}, as well as 250-ml shake flasks containing 25 ml of medium supplemented with 10 g of xylulose or 20 g of xylose liter^{-1} were incubated in a rotary shaker at 30°C. The growth rate was determined by measuring the optical density at 620 nm. Duplicate cultivation experiments were performed for each substrate and strain.

RESULTS

Chemostat cultivation of *S. cerevisiae* strains TMB 3399 and TMB 3400. *S. cerevisiae* TMB 3399 and TMB 3400 were first cultivated in a batch, with the carbon source consisting of 10 g of glucose liter^{-1}, and from which the maximum specific growth rates were determined to be 0.44 ± 0.006 h^{-1} for both strains. At the late exponential phase, chemostat cultivation was initiated by feeding with a defined mineral medium, first supplemented with glucose (10 g liter^{-1}) and, six volume changes later, with a mixture of 10 g of glucose plus 10 g of xylose liter^{-1}. Since only *S. cerevisiae* TMB 3400 and not *S. cerevisiae* TMB 3399 shows a maximum specific growth rate exceeding 0.1 h^{-1} on xylose, which is required to prevent washout, only *S. cerevisiae* TMB 3400 was cultivated in chemostat with xylose as the sole carbon source. *S. cerevisiae* TMB 3400 was first cultivated in a batch supplemented with 20 g of xylose liter^{-1}, after which a feed of 20 g of xylose liter^{-1} was added.

The biomass yield was ca. 0.47 g of biomass g of consumed carbohydrate^{-1} for both strains during growth on glucose and glucose plus xylose, whereas it decreased to 0.43 g biomass g of consumed carbohydrate^{-1} for *S. cerevisiae* TMB 3400 during growth on xylose only (Table 1). The specific glucose consumption was 247 and 231 mg g of biomass^{-1} h^{-1} for *S. cerevisiae* TMB 3399 and *S. cerevisiae* TMB 3400, respectively, when cultivated on glucose only. When grown on a mixture of glucose and xylose, *S. cerevisiae* TMB 3399 gave higher residual glucose and xylose concentrations, 0.09 and 4.6 g liter^{-1}, respectively, than *S. cerevisiae* TMB 3400, which gave no detectable glucose and only 2.9 g of xylose liter^{-1}. The specific glucose consumption was higher for *S. cerevisiae* TMB 3399: 160 mg g of biomass^{-1} h^{-1} compared to 142 mg g of biomass^{-1} h^{-1} for *S. cerevisiae* TMB 3400. The reverse was true for the specific xylose consumption: 85 mg g of biomass^{-1} h^{-1} for *S. cerevisiae* TMB 3399 and 97 mg g of biomass^{-1} h^{-1} for *S. cerevisiae* TMB 3400. A total of 2.5 mg g of biomass^{-1} h^{-1} xylitol was formed during the cultivation of *S. cerevisiae* TMB 3399. During growth on xylose only, *S. cerevisiae* TMB 3400 consumed 12.3 g liter^{-1} of 20.8 g liter^{-1} in the feed and the specific xylose uptake, 254 mg g of biomass^{-1} h^{-1}, more than doubled in comparison to cultivation on the mixture of glucose and xylose. A K_s value of 4.9 g liter^{-1}, or 33 mM, was obtained by employing Monod kinetics (16), using $D = 0.1$ h^{-1}, $D_{crit} = 0.14$ h^{-1}, and s = 12.3 g liter^{-1}. *S. cerevisiae* TMB 3400 also produced a small amount of xylitol, 1 mg g of biomass^{-1} h^{-1}. The carbon balances closed within 94 to 103% for all fermentation experiments.

mRNA expression levels. The mRNA expression levels of the more than 6,000 genes in the genome of *S. cerevisiae* were monitored simultaneously by using DNA microarrays. In the present investigation, we chose to analyze the mRNA expression levels of the genes encoding sugar transporters and the enzymes involved in xylose metabolism, the pentose phosphate pathway, glycolysis, gluconeogenesis, and galactose metabolism (Table 2), as well as the genes involved in regulation (Table 3). All microarray experiments were performed in duplicate with cells from two independent fermentation experiments.

There are 20 hexose transporters in *S. cerevisiae* (2), of which the mRNA expression levels for *HXT4*, *HXT5*, and *HXT7*, together with galactose permease, *GAL2*, changed during the different cultivation conditions and between the strains (Table 2). *HXT5* was more highly expressed in *S. cerevisiae* TMB 3400 than in *S. cerevisiae* TMB 3399. The expression of *GAL2* was about 100 times higher when xylose was the only sugar in the feed than when glucose was also present.

With regard to xylose metabolism, the heterologous genes encoding XR and XDH were not included in the analysis, since they were cloned from *P. stipitis* and therefore are not represented on the *S. cerevisiae* DNA microarrays. The expression of *S. cerevisiae* genes encoding enzymes with XR (YHR104w) (9) and XDH (YLR070c) (19) activities was analyzed. These two genes showed slightly increased expression during growth on only xylose. The *XKS1* gene encoding XK was expressed at levels about twice as high in *S. cerevisiae* TMB 3400 as those in TMB 3399.

SOL3 and *GND1*, as well as *TAL1* and *TKL1*, encoding enzymes in the oxidative and nonoxidative pentose phosphate pathway, respectively, were expressed at higher levels in *S. cerevisiae* TMB 3400 than in TMB 3399. Among the genes encoding glycolytic enzymes, *HXK1*, *HXK2*, *ENO2* and, possibly, YDR516c showed lower expression levels when TMB 3400 was grown on only xylose. Genes encoding the gluconeogenetic enzyme phosphoenolpyruvate carboxykinase and the glyoxalate enzyme isocitrate lyase, as well as genes involved in galactose metabolism, were upregulated in *S. cerevisiae* TMB 3400 during growth on xylose only.

Among the genes involved in regulation, notably YBR083W and YPR199C were expressed at lower levels in *S. cerevisiae* TMB 3400 than in TMB 3399 (Table 3). The *PET18* gene (YCR020C), which encodes a transcription regulator, was expressed at high levels in *S. cerevisiae* TMB 3400 during growth on the mixture of glucose and xylose. When *S. cerevisiae* TMB 3400 was grown on xylose only, the expression of *MIG1* and *MIG2* was lower, whereas the expression of *CAT8* was higher.

Enzymatic activities. The specific activities of enzymes involved in the initial xylose metabolism, i.e., XR, XDH, and XK, are given in Table 4. All three enzymes displayed higher activities in *S. cerevisiae* TMB 3400 than in *S. cerevisiae* TMB 3399 regardless of whether the feed contained only glucose or glucose plus xylose. The specific activity determined in the cell

TABLE 2. mRNA expression levels in *S. cerevisiae* strains TMB 3399 and TMB 3400 for genes coding for transporters, enzymes involved in xylose metabolism, the pentose phosphate pathway, glycolysis, glyoxylate shunt, gluconeogenesis, and galactose metabolism

Type and open reading frame	Gene	Description	Mean mRNA levels (arbitrary units) ± SD[a]				
			Glucose		Glucose + xylose		Xylose (TMB 3400)
			TMB 3399	TMB 3400	TMB 3399	TMB 3400	
Hexose transporters							
YHR092C	*HXT4*	High-affinity glucose transporter	37 ± 11	25 ± 3	139 ± 104	55 ± 2	8 ± 1
YHR096C	*HXT5*	Hexose transporter	634 ± 75	**1,038 ± 176**	654 ± 145	**883 ± 157**	**1,314 ± 218**
YDR342C	*HXT7*	Hexose transporter	1,967 ± 211	2,097 ± 124	2,476 ± 290	1,981 ± 249	1,327 ± 36
YLR081W	*GAL2*	Galactose permease	11 ± 3	6 ± 2	8 ± 5	8 ± 7	**688 ± 19**
Xylose metabolism							
YHR104W	*GRE3*	Aldo-keto-reductase	160 ± 12	209 ± 18	209 ± 50	249 ± 14	306 ± 68
YLR070C		Strong similarity to sugar dehydrogenases	55 ± 8	47 ± 5	60 ± 7	58 ± 12	78 ± 3
YGR194C	*XKS1*	Xylulokinase	613 ± 87	**1,278 ± 102**	773 ± 160	**1,207 ± 23**	**1,605 ± 176**
Pentose phosphate pathway							
YNL241C	*ZWF1*	Glucose-6-phosphate dehydrogenase	480 ± 69	400 ± 55	458 ± 69	471 ± 14	359 ± 32
YHR163W	*SOL3*	Shows similarity to glucose-6-phosphate dehydrogenase non-catalytic domains	930 ± 169	**1,577 ± 168**	932 ± 95	**1,824 ± 32**	**1,739 ± 168**
YHR183W	*GND1*	Phosphogluconate dehydrogenase (decarboxylating)	1,664 ± 210	**2,383 ± 104**	1,804 ± 101	**2,371 ± 114**	**2,396 ± 107**
YOR095C	*RKI1*	Ribose-5-phosphate ketol-isomerase	130 ± 4	207 ± 38	124 ± 18	129 ± 7	159 ± 27
YJL121C	*RPE1*	D-Ribulose-5-phosphate 3-epimerase	739 ± 13	707 ± 35	757 ± 181	683 ± 57	342 ± 38
YLR354C	*TAL1*	Transaldolase	885 ± 66	**1,124 ± 72**	1,018 ± 129	**1,209 ± 28**	**1,213 ± 13**
YPR074C	*TKL1*	Transketolase 1	1,191 ± 34	**1,595 ± 28**	1,400 ± 63	**1,646 ± 209**	**1,428 ± 67**
Glycolysis							
YFR053C	*HXK1*	Hexokinase I (PI) (also called hexokinase A)	1,250 ± 36	1,299 ± 261	1,440 ± 40	1,267 ± 194	700 ± 8
YGL253W	*HXK2*	Hexokinase II (PII) (also called hexokinase B)	890 ± 36	782 ± 69	873 ± 149	877 ± 194	317 ± 23
YCL040W	*GLK1*	Glucokinase	1,600 ± 100	1,771 ± 317	2,286 ± 249	1,648 ± 36	2,019 ± 125
YDR516C		Strong similarity to glucokinase	662 ± 44	629 ± 69	735 ± 32	785 ± 44	474 ± 100
YBR196C	*PG1*	Glucose-6-phosphate isomerase	1,349 ± 65	1,538 ± 114	1,640 ± 26	1,427 ± 97	1,400 ± 25
YGR240C	*PFK1*	Phosphofructokinase alpha subunit	1,078 ± 56	1,157 ± 170	1,217 ± 89	1,085 ± 51	1,254 ± 49
YKL060C	*FBA1*	Aldolase	3,244 ± 580	3,830 ± 206	4,123 ± 31	3,561 ± 550	4,194 ± 225
YDR050C	*TPI1*	Triosephosphate isomerase	2,743 ± 483	3,196 ± 184	3,324 ± 243	3,370 ± 426	3,452 ± 21
YJL052W	*TDH1*	Glyceraldehyde-3-phosphate dehydrogenase 1	2,924 ± 600	3,156 ± 74	3,288 ± 30	3,056 ± 516	3,397 ± 255
YJR009C	*TDH2*	Glyceraldehyde-3-phosphate dehydrogenase 2	3,151 ± 466	3,856 ± 76	4,128 ± 11	3,193 ± 623	3,861 ± 115
YGR192C	*TDH3*	Glyceraldehyde-3-phosphate dehydrogenase 3	4,058 ± 1066	4,591 ± 250	4,451 ± 45	4,571 ± 749	4,628 ± 553
YCR012W	*PGK1*	3-Phosphoglycerate kinase	2,983 ± 507	3,573 ± 123	3,616 ± 58	3,560 ± 507	3,852 ± 217
YKL152C	*GPM1*	Phosphoglycerate mutase	3,206 ± 582	3,371 ± 332	3,796 ± 197	3,248 ± 429	4,037 ± 220
YGR254W	*ENO1*	Enolase I	4,015 ± 656	4,410 ± 107	4,498 ± 21	4,278 ± 373	4,152 ± 242
YHR174W	*ENO2*	Enolase II	2,902 ± 191	3,402 ± 102	3,441 ± 264	2,995 ± 369	2,527 ± 71
YAL038W	*PYK1*	Pyruvate kinase 1	3,051 ± 379	3,235 ± 35	3,617 ± 39	3,307 ± 384	3,479 ± 187
Gluconeogenesis/glyoxylate shunt							
YKR097W	*PCK1*	Phosphoenolpyruvate carboxykinase	381 ± 124	239 ± 94	252 ± 26	193 ± 77	**694 ± 9**
YER065C	*ICL1*	Isocitrate lyase	454 ± 25	396 ± 54	473 ± 91	365 ± 140	**1,199 ± 62**
Galactose metabolism							
YBR020W	*GAL1*	Galactokinase	6 ± 0	3 ± 0	4 ± 2	4 ± 1	**160 ± 22**
YBR018C	*GAL7*	Galactose-1-phosphate uridyl transferase	8 ± 2	5 ± 0	9 ± 1	8 ± 1	**477 ± 2**
YBR019C	*GAL10*	UDP-glucose 4-epimerase	11 ± 1	10 ± 1	14 ± 1	13 ± 0	**333 ± 49**

[a] Values are means of two independent fermentation experiments. High mRNA expression levels in TMB 3400 are indicated in boldface.

extracts from xylose-grown *S. cerevisiae* TMB 3400 was very low for all three enzymes.

Characterization of *PET18*. Among the genes encoding proteins involved in gene regulation that were differently expressed in *S. cerevisiae* TMB 3399 and its mutant *S. cerevisiae* TMB 3400 (Table 3), the effect of the transcription regulator encoded by *PET18* on xylose utilization was further investigated. Strains with overexpression and deletion of *PET18*, as well as control strains, were developed and cultivated in shake flasks with a defined mineral medium supplemented with glu-

TABLE 3. mRNA levels of genes involved in regulation

Open reading frame	Gene	Description	Mean mRNA levels (arbitrary units) ± SD[a]				
			Glucose		Glucose + xylose		Xylose (TMB 3400)
			TMB 3399	TMB 3400	TMB 3399	TMB 3400	
YBR083W	*TEC1*	Transcription factor of the TEA/ATTS DNA-binding domain family, regulator of Ty1 expression	140 ± 17	**68 ± 15**	175 ± 37	**88 ± 31**	**107 ± 4**
YPR199C	*ARR1*	Similar to transcriptional regulatory elements *YAP1* and *CAD1*	160 ± 20	**114 ± 12**	166 ± 16	**109 ± 2**	**120 ± 11**
YCR020C	*PET18*	Transcription regulator	37 ± 1	41 ± 11	77 ± 32	**204 ± 118**	23 ± 11
YDR477W	*SNF1*	Protein serine/threonine kinase	94 ± 19	109 ± 1	64 ± 1	98 ± 1	116 ± 2
YGL115W	*SNF4*	Associates with Snf1p	175 ± 25	206 ± 1	145 ± 3	179 ± 17	177 ± 1
YGL035C	*MIG1*	C2H2 zinc finger protein, which resembles the mammalian Egr and Wilms tumor proteins	14 ± 7	14 ± 3	23 ± 0	10 ± 9	**6 ± 2**
YGL209W	*MIG2*	Protein containing zinc fingers very similar to the zinc fingers in Mig1p	21 ± 1	16 ± 2	28 ± 1	22 ± 3	**14 ± 6**
YMR280C	*CAT8*	Zinc cluster protein involved in activating gluconeogenic genes; related to Gal4p	62 ± 1	58 ± 12	66 ± 9	48 ± 15	**129 ± 23**
YPL248C	*GAL4*	Zinc finger transcription factor of the Zn(2)-Cys(6) binuclear cluster domain type	66 ± 2	59 ± 0	66 ± 0	74 ± 0	57 ± 2
YML051W	*GAL80*	Regulatory protein	188 ± 12	147 ± 3	190 ± 27	174 ± 6	182 ± 17
YDR009W	*GAL3*	Galactokinase	51 ± 17	44 ± 5	56 ± 9	39 ± 4	80 ± 11

[a] Expression levels where TMB 3400 differs greatly are indicated in boldface.

cose, xylose, xylulose, and ethanol (Table 5). The effect of *PET18* on glucose and ethanol growth was negligible. The growth on xylulose was not affected significantly when *PET18* was overexpressed, but deletion of the gene slightly improved the growth rate. Xylose growth was hampered by overexpression, but the deletion did not change the performance of the strain.

DISCUSSION

S. cerevisiae TMB 3399, transformed with the genes encoding XR, XDH, and XK, showed a growth rate of 0.025 h^{-1} (29a) and was subjected to chemical mutagenesis with ethyl methanesulfonate to further improve its ability to utilize xylose. The best-performing mutant, *S. cerevisiae* TMB 3400, showed a >5-fold increase in maximum specific growth on xylose (0.14 h^{-1}) (29a). Random mutagenesis combined with a good selection protocol generates strains that display the characteristics selected for, although the site(s) of the mutation(s) remains unknown. In the present investigation we report, for the first time, the use of microarray technology to characterize a recombinant strain of *S. cerevisiae* that is chemically mutated for better growth on a nonnatural substrate. All microarray experiments were performed in duplicate, starting with independent fermentation experiments, and the percentage error (difference from the mean as a fraction of the mean) generally was less than 20%. Thus, reproducible DNA microarray results were obtained for a diploid, industrial strain of *S. cerevisiae.*

In *S. cerevisiae* TMB 3400, the genes *HXT5* (encoding a hexose transporter), *XKS1* (encoding XK), and *SOL3* and *GND1* (coding for proteins in the oxidative pentose phosphate pathway), as well as, but to a lesser extent, *TKL1* and *TAL1* in the nonoxidative pentose phosphate pathway, were expressed at higher levels than in the parental strain *S. cerevisiae* TMB 3399. The higher expression of these genes could explain the improved growth rate of *S. cerevisiae* TMB 3400 on xylose, as well as its higher xylose uptake from a feed containing a mixture of xylose and glucose.

The *HXT5* gene encodes a hexose transporter with moderate affinity for glucose (40 mM) (5). A previous investigation (5) reported abundant *HXT5* expression during slow growth on glucose and during growth on nonfermentable carbon sources but, in contrast to our results, this was suggested to be glucose repressed.

When TMB 3400 and TMB 3399 were compared under the same conditions, either with glucose or with the glucose-xylose mixture, the higher *XKS1* mRNA expression levels monitored for TMB 3400 (Table 2) agreed well with the higher XK activities measured in the cell extracts (Table 4). That the XK activity in *S. cerevisiae* TMB 3400 was higher than in TMB 3399 is also in agreement with a previous investigation of an *S. cerevisiae* strain mutated for improved xylose growth. The enzymatic activity of XK in the mutated *S. cerevisiae* IM2, with a maximum specific growth rate of 0.08 h^{-1}, was 60% higher than in its parent *S. cerevisiae* H that has a maximum specific growth rate of 0.03 h^{-1} (24). Furthermore, XK activities were 5 to 10 times higher in mutants of the natural xylose-utilizing yeasts *Pachysolen tannophilus* (11) and *Candida utilis* (12), which were selected for improved utilization of xylose and xylitol, respectively.

The higher expression of *HXT5*, *XKS1*, *SOL3*, and *GND1*, as well as of *TKL1* and *TAL1*, in *S. cerevisiae* TMB 3400 could be due to an altered expression of one or several transcription

TABLE 4. Enzymatic activities of XR, XDH, and XK from chemostat cultivation of *S. cerevisiae* strains TMB 3399 and TMB 3400 on various feed concentrations of glucose and xylose

S. cerevisiae strain	Feed concn ($g\ liter^{-1}$) on:		Enzymatic activity ($U\ mg$ of $protein^{-1}$)		
	Glucose	Xylose	*XR*	*XDH*	*XK*
TMB 3399	10.8 ± 0.2	0	0.20 ± 0.01	0.33 ± 0.02	0.44 ± 0.04
TMB 3400	10.8 ± 0.2	0	0.50 ± 0.02	0.83 ± 0.1	0.60 ± 0.18
TMB 3399	10.2 ± 0.1	10.0 ± 0.2	0.13 ± 0.01	0.36 ± 0.1	0.20 ± 0.08
TMB 3400	10.2 ± 0.1	10.0 ± 0.2	0.32 ± 0.07	0.75 ± 0.25	0.27 ± 0.02
TMB 3400	0	20.8 ± 0.1	0.08 ± 0.01	0.22 ± 0.07	0.07 ± 0.01

TABLE 5. Maximum specific growth rates of *S. cerevisiae* strains TMB 3152, TMB 3155, TMB 3159, and TMB 3160 grown in shake flasks in a defined mineral medium supplemented with glucose, ethanol, xylulose, and xylose

S. cerevisiae strain	Relevant genotype	Maximum specific growth rate (μ_{max} [h^{-1}])[a]			
		Glucose	Xylose	Xylulose	Ethanol
TMB 3155	Overexpressed YCR020c	0.35	0.01	0.05	0.06
TMB 3152	Reference	0.34	0.02	0.05	0.05
TMB 3160	Deleted YCR020c	0.35	0.07	0.06	0.04
TMB 3159	Reference	0.37	0.07	0.04	0.03

[a] Glucose, 20 g liter^{-1}; xylose, 10 g liter^{-1}; Xylulose, 10 g liter^{-1}; ethanol, 10 g liter^{-1}. Values are means of duplicate experiments in which the relative error was less than 2%

regulators acting on these genes. The transcription factors coded for by YCR020C, YBR083W, and YPR199C were differently expressed in *S. cerevisiae* TMB 3400 and TMB 3399. The open reading frame YCR020C encodes the transcription factor *Pet18p*, which was expressed at higher levels in *S. cerevisiae* TMB 3400 than in *S. cerevisiae* TMB 3399 during growth on the mixture of glucose and xylose. The decision to investigate the influence of *PET18* on xylose utilization was based on its reported effect on carbon utilization. Mutants defective in *PET18* have been reported to be unable to grow on nonfermentable carbon sources (30). However, the strain in which *PET18* was deleted was capable of growth on ethanol. Furthermore, xylose growth seemed to be unaffected by deleting and by overexpressing *PET18*. The transcription factors encoded by YBR083W and YPR199C deserve further attention and their effect on xylose utilization in *S. cerevisiae* will be reported in a forthcoming study.

The development of the superior xylose-utilizing *S. cerevisiae* TMB 3400 allowed us to perform a physiological comparison, as well as a comparison of mRNA expression levels, between growth levels on xylose and glucose. The first chemostat cultivation of a recombinant *S. cerevisiae* strain on xylose as the sole carbon source is presented here. *S. cerevisiae* TMB 3400 consumed ca. 40% of the xylose in the feed. This permitted us to determine the K_s value for xylose (33 mM), which is ca. 60 times higher than the corresponding value reported for growth on glucose (0.55 mM) (27). The biomass yield was lower on xylose than on glucose; therefore, *S. cerevisiae* TMB 3400 apparently cannot obtain as much energy from xylose as from glucose, which points toward a difference in the metabolism of these substrates. The gluconeogenetic gene encoding phosphoenolpyruvate carboxykinase and the glyoxylate gene encoding isocitrate lyase were upregulated during growth on xylose. When carbon is channeled through the glyoxylate shunt, ATP (GTP) is lost due to a reduced flux between succinyl coenzyme A and succinate. A lower ATP yield may be responsible for the lower biomass yield of *S. cerevisiae* TMB 3400 on xylose than on glucose. In a previous investigation, batch cultivation of *S. cerevisiae* TMB 3399 and 3400 showed biomass yields of 0.39 and 0.41 g of biomass g of xylose^{-1}, respectively (29a). The fact that both the transformant *S. cerevisiae* TMB 3399 and the mutant *S. cerevisiae* TMB 3400 displayed similar biomass yields on xylose indicates that the lower biomass yield on xylose is not due to a mutation. The genes encoding phosphoenolpyruvate carboxykinase and isocitrate lyase are upregulated by *CAT8* (3, 17, 18), which in turn is repressed by the glucose transcription regulator *Mig1p* (8). Our results show higher mRNA levels of *CAT8* and lower expression of *MIG1* during growth on xylose in comparison to growth on glucose.

MIG1 also regulates genes involved in galactose metabolism. The analysis of *S. cerevisiae* TMB 3400 showed that galactose permease, galactokinase, galactose 1-phosphate uridyl transferase and UDP-glucose 4-epimerase were expressed at higher levels during growth on only xylose in comparison to growth on glucose or on a combination of glucose and xylose. In the presence of glucose, *MIG1* causes repression of the transcription of genes involved in galactose metabolism (15). However, galactose has been reported to be necessary for the full initiation of transcription (23, 32), and it remains to be elucidated whether the increased expression of these genes in *S. cerevisiae* TMB 3400 is mediated by xylose or is due to a mutation.

Some of the genes encoding glycolytic enzymes, notably *HXK2*, showed lower expression levels when TMB 3400 was grown on only xylose. It has been demonstrated that the induction of glycolytic genes requires increased concentrations of metabolites in the early stages of glycolysis (7, 13, 20). These concentrations might be insufficient for the full induction of glycolysis during xylose growth.

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