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Deletion of the *GRE3* Aldose Reductase Gene and Its Influence on Xylose Metabolism in Recombinant Strains of *Saccharomyces cerevisiae* Expressing the *xylA* and *XKS1* Genes

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***Saccharomyces cerevisiae* ferments hexoses efficiently but is unable to ferment xylose. When the bacterial enzyme xylose isomerase (XI) from *Thermus thermophilus* was produced in *S. cerevisiae,* xylose utilization and ethanol formation were demonstrated. In addition, xylitol and acetate were formed. An unspecific aldose reductase (AR) capable of reducing xylose to xylitol has been identified in *S. cerevisiae*. The *GRE3* gene, encoding the AR enzyme, was deleted in *S. cerevisiae* CEN.PK2-1C, yielding YUSM1009a. XI from *T. thermophilus* was produced, and endogenous xylulokinase from *S. cerevisiae* was overproduced in *S. cerevisiae* CEN.PK2-1C and YUSM1009a. In recombinant strains from which the *GRE3* gene was deleted, xylitol formation decreased twofold. Deletion of the *GRE3* gene combined with expression of the *xylA* gene from *T. thermophilus* on a replicative plasmid generated recombinant xylose utilizing *S. cerevisiae* strain TMB3102, which produced ethanol from xylose with a yield of 0.28 mmol of C from ethanol/mmol of C from xylose. None of the recombinant strains grew on xylose.**

Ethanol production from renewable lignocellulosic material represents an environmentally sustainable alternative to fossil-derived gasoline. In most lignocellulosic material, the second-most-common sugar is xylose (13). For an economically feasible fuel production process, both hexose and pentose sugars must be fermented to form ethanol (35). The yeast *Saccharomyces cerevisiae* is robust and well adapted for ethanol production, but it is unable to produce ethanol from xylose.

The initial metabolism of xylose in natural xylose-utilizing yeasts such as *Pichia stipitis* is catalyzed by xylose reductase (XR), which reduces xylose to xylitol, and xylitol dehydrogenase (XDH), which oxidizes xylitol to xylulose. Xylulose is then phosphorylated by xylulokinase (XK) to xylulose-5-phosphate that is further metabolized in the pentose phosphate pathway. *P. stipitis* requires low and carefully controlled oxygenation (28) and is sensitive to ethanol (8), which limits its use for industrial ethanol production. Recombinant *Saccharomyces* strains producing XR and XDH from *P. stipitis* in addition to overexpression of the homologous *XKS1* gene encoding XK produce ethanol from xylose, with xylitol as a major by-product (9, 14, 36).

In bacteria, xylose isomerase (XI), encoded by the *xylA* gene, catalyzes the isomerization of xylose to xylulose. *xylA* genes from several bacteria have been cloned and transformed into *S. cerevisiae*, including *xylA* from *Actinoplanes missouriensis* (1), *Bacillus subtilis* (1), *Clostridium thermosulfurigenes* (19), *Escherichia coli* (15, 23), and *Streptomyces rubiginosus* (24). The use of rich-medium ethanol formation from xylose has been reported in recombinant *Schizosaccharomyces pombe* expressing *xylA* from *E. coli* (6). Ethanol formation from xylose in synthetic complete (SC) medium has so far been demonstrated only in recombinant *S. cerevisiae* expressing the *xylA* gene from *Thermus thermophilus* (37). A major by-product was xylitol, which inhibits XI (39) (Fig. 1). *S. cerevisiae* produces an unspecific aldose reductase (AR), encoded by the *GRE3* gene on chromosome VIII (11), capable of reducing xylose to xylitol (17).

In the present study, the *GRE3* gene was deleted from chromosome VIII, yielding a recombinant *S. cerevisiae* strain with reduced xylitol formation. XI was introduced into the *S. cerevisiae* reference strain and in the Δ*gre3* strain to investigate the inhibition of XI by xylitol during fermentation of xylose. XK was overproduced in strains expressing *xylA* to increase the flux of xylulose into the central metabolism. The effects of glucose and oxygen supplementation were also studied.

MATERIALS AND METHODS

Strains and plasmids. The genotypes of the microbial strains and plasmids used in the present study are summarized in Table 1. Plasmids were constructed and used to transform *E. coli* DH5α or JM109. Plasmid pUSM1006 was used to transform *S. cerevisiae* CEN.PK2-1C. Plasmids pBXI and pXks were used to transform YUSM1009a and CEN.PK2-1C. All strains were stored at −80°C.

Culture conditions. *S. cerevisiae* CEN.PK2-1C was precultured in yeast extract-peptone-dextrose (26) or synthetic medium (20 g of glucose/liter and 6.7 g of yeast nitrogen base [YNB] without amino acids [Difco, Detroit, Mich.]/liter) supplemented with amino and nucleic acids (20 mg of tryptophan, uracil, and histidine per liter and 30 mg of leucine/liter, omitting those used as selection markers). The YNB medium was also used to make 5-fluoroorotic acid (5-FOA)-agar plates containing 1 g of 5-FOA (Sigma, Stockholm, Sweden)/liter for the selection of transformants that lost the *URA3* marker with replica plating (38). In fermentation, a defined mineral medium (34) supplemented with amino acids including vitamins, trace elements, and citric acid buffer, pH 5.5, was used. The

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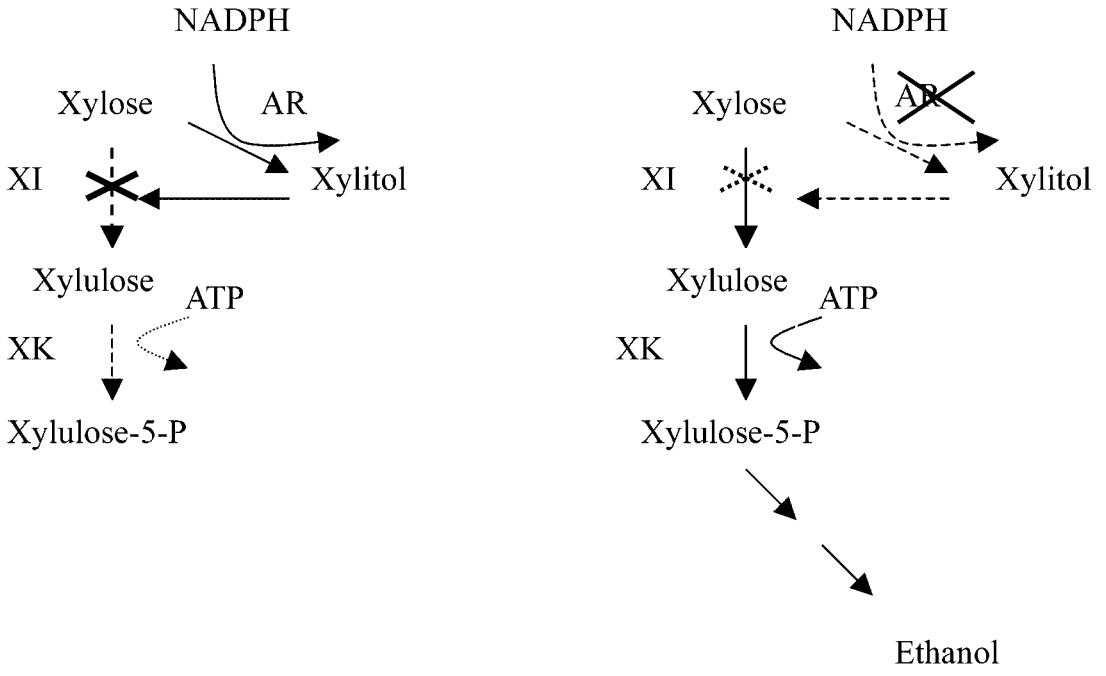

FIG. 1. Model of xylose metabolism in recombinant *S. cerevisiae* expressing XI with (A) and without (B) the *GRE3* gene.

medium also contained 50 g of xylose/liter and 20 g of glucose/liter where appropriate. Bacterial strains were grown in Luria-Bertani medium (2), and transformants were selected with ampicillin (50 μg ml^{-1}) after growth at 37°C. When cells were grown on solid media, 20 g of agar/liter was added.

DNA manipulations and amplifications. Standard techniques for nucleic acid manipulations were used (22). Plasmids were prepared using a Qiagen Mini plasmid purification kit (Qiagen GmbH, Hilden, Germany). Restriction enzymes and other modifying enzymes were from Boehringer Mannheim Scandinavia AB (Bromma, Sweden). Plasmid transformations of *E. coli* were performed with the calcium chloride method (22). Yeast transformations were performed by the lithium acetate method (12).

The genomic DNA of *S. cerevisiae* CEN.PK2-1C was used as template for the PCR of the corresponding *GRE3* promoter and terminator sequences. The oligonucleotides used for amplifying the promoter region were ARpL (left

TABLE 1. Microbial strains and plasmids used in this study

Strain or plasmid	Relevant feature(s)	Source or reference
Strains		
E. coli		
JM109	F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} Δ(*lacZ*)M15 Δ(*lac-proAB*) *supE44 hsdR17 recA1 gyrA96 thi-1 endA1 relA1* e14$^-$ λ$^-$	40
DH5α	F$^-$ ϕ80d*lacZ* ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17* (r_K^+ m_K^-) *phoA supE44* λ$^-$ *thi-1 gyrA96 relA1*	Life Technologies, Rockville, Md.
S. cerevisiae		
CEN.PK2-1C	*MAT***a** *leu2-3,112 his3-Δ1 ura3-52 trp1-289 MAL2-8*(Con) *MAL3 SUC3*	10
YUSM1006a	CEN.PK2-1C(pUSM1006)	This work
YUSM1009a	CEN.PK2-1C (Δ*gre3*)	This work
TMB3101	CEN.PK2-1C (*xylA LEU2*)	This work
TMB3102	YUSM1009a (Δ*gre3 xylA LEU2*)	This work
TMB3103	CEN.PK2-1C (*xylA LEU2 XKS1 TRP1*)	This work
TMB3104	YUSM1009a (Δ*gre3 xylA LEU2 XKS1 TRP1*)	This work
Plasmids		
pBR322	*bla tet*	3
pGEM-T easy vector	*bla*	Promega Corporation, Madison, Wis.
pBluescript SK(−)	*bla*	GenBank (accession no. 52330)
pBXI	*bla PGKp:xylA:PGKt LEU2*	37
pXks	*bla PGKp:XKS1:PGKt TRP1*	9
pUSM1002	*bla GRE3t* in pBR322	This work
pUSM1003	*GRE3p* in pGEM-T easy	This work
pUSM1004	*bla GRE3p*	This work
pUSM1006	*bla URA3 GRE3p GRE3t*	This work
pUSM1007	pUC8/*URA3*	This work
pUSM1008	*bla GRE3t* in pBluescript SK(−)	This work

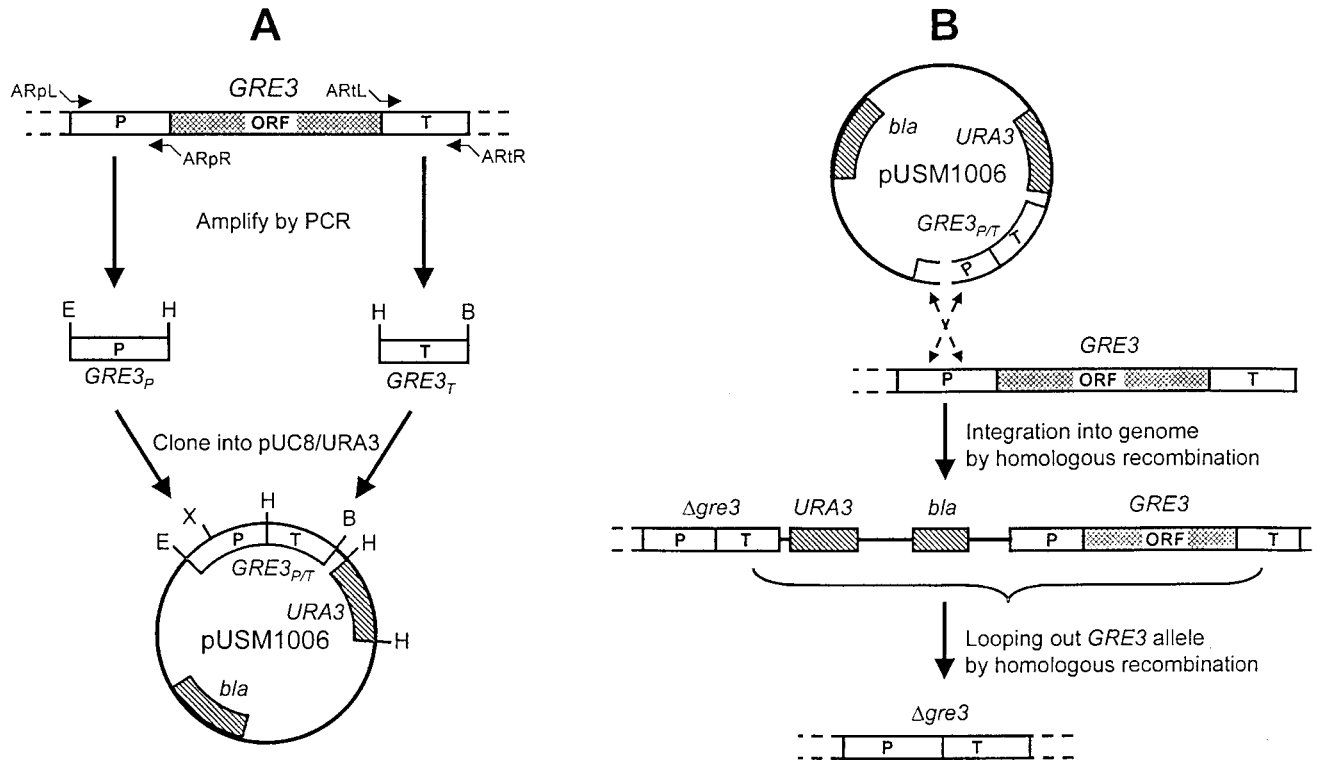

FIG. 2. (A) Schematic summary of the construction of plasmid pUSM1006. The 5′ promoter (P) and 3′ terminator (T) regions of the corresponding *GRE3* gene were amplified using oligonucleotide pairs ARpL-ARpR and ARtL-ARtR, respectively. The selectable markers *bla* and *URA3* are indicated on the plasmid by striped boxes, and the *GRE3* promoter ($GRE3_P$) and terminator ($GRE3_T$) sequences are indicated by open boxes. Sites for restriction endonucleases are indicated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I. (B) The plasmid pUSM1006 was linearized by cutting plasmid pUSM1006 with *Xba*I and integrating it into the genome by homologous recombination. The *GRE3* allele was subsequently looped out by homologous recombination between the *GRE3* terminator sequences. ORF, open reading frame.

primer) (5′-GAT CGA ATT CTT TGT AAC TGT AAT TTC ACT CAT GC-3′ [*Eco*RI is underlined]) and ARpR (right primer) (5′-GAT CAA GCT TAA TCC ATA CTC AAC GAC CAT ATG-3′ [*Hin*dIII is underlined]). To amplify the terminator region, the primers used were ARtL (left primer) (5′-GTA CAA GCT TTT TCC AAT TTT ATT TTA CGA TTT-3′ [*Hin*dIII is underlined]) and ARtR (right primer) (5′-GTA AGG ATC CGC TCA TAT CTT GCT GTT G-3′ [*Bam*HI is underlined]). These PCR primers were based on the published sequence of the *GRE3* promoter and terminator regions of *S. cerevisiae*. This information was found at the Saccharomyces Genome Database website (http://genome-www.stanford.edu/Saccharomyces/). All primers contained a restriction endonuclease site (Fig. 2A). For amplification of the DNA, *Pfu* polymerase (Stratagene, Capetown, Republic of South Africa) was used. The PCR mix contained PCR buffer with 2 mM $MgSO_4$, 2 mM deoxynucleoside triphosphate, 0.5 μM concentrations of each primer, 0.1 μg of template, and 2.5 U of *Pfu* polymerase enzyme in a final volume of 50 μl. The thermocycler (Eppendorf 5330 plus; Analytical Instrument Recycle, Inc., Golden, Colo.) was used under the following conditions: 95°C for 5 min; 25 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 1 min; 10 min at 72°C. Then the mixture was chilled to 4°C.

Southern blot hybridization. Total DNA was isolated from *S. cerevisiae* YUSM1006a and putative YUSM1009a, digested with *Hin*dIII, separated on a 1% agarose gel, and transferred to a Hybond-N membrane (Amersham Sweden AB, Stockholm, Sweden). Southern hybridizations (29) were performed as described by Sambrook et al. (22). The 0.4-kb promoter DNA region of *GRE3* was used as the ^{32}P-labeled probe.

Preparation of crude cell extracts for enzyme measurements. Yeast cells were grown at 30°C in a YNB medium containing the required amino acids, 20 g of glucose/liter, and 50 g of xylose/liter. The cells were harvested in the stationary phase by centrifugation at 3,000 × *g* for 5 min and washed in 0.9% NaCl. The pellet was resuspended in disintegration buffer (100 mM triethanolamine [pH 7], 1.0 mM phenylmethylsulfonyl fluoride in dimethyl sulfoxide) and vortexed twice for 5 min at 4°C with an equal volume of glass beads (0.5 mm in diameter). The disintegrated cell mixture was centrifuged at 5,000 × *g* for 5 min at 4°C, and the

TABLE 2. Specific AR, XI, and XK activities in strains used in the present investigation

Strain	Genotype[a]	AR activity (mU mg^{-1}) (mean ± SD)	XI activity (U mg^{-1}) (mean ± SD)	XK activity (U mg^{-1}) (mean ± SD)
CEN.PK2-1C	Ref	4.5 ± 0.5	<0.05	<0.01
TMB3101	Ref plus *xylA*	4.3 ± 0.4	0.90 ± 0.16	<0.01
TMB3103	Ref plus *xylA* plus *XKS1*	4.6 ± 0.5	0.55 ± 0.22	14 ± 1.5
YUSM1009a	Δ*gre3*	<1	<0.05	<0.01
TMB3102	Δ*gre3* plus *xylA*	<1	1.01 ± 0.28	0.38 ± 0.12
TMB3104	Δ*gre3* plus *xylA* plus *XKS1*	<1	0.42 ± 0.13	11 ± 1.1

[a] Ref, reference strain.

TABLE 3. Carbon balances after fermentation of 50 g of xylose/liter[a]

Strain	Amt of C (mmol) (mean ± SD) from:						Carbon recovery (%)	$Yield_{ethanol/xylose}$ (mmol of C from ethanol/mmol of C from xylose) (mean ± SD)
	Xylose (substrate)	Ethanol	Xylitol	Glycerol	Acetic acid	CO_2[b]		
CEN.PK2-1C	36 ± 3	ND[c]	20 ± 1	0.62 ± 0.07	3.8 ± 0.03	1.9	75	0
TMB3101	37 ± 4	ND	21 ± 5	0.52 ± 0.08	3.2 ± 0.01	1.6	72	0
TMB3103	31 ± 7	ND	16 ± 4	0.27 ± 0.05	3.6 ± 0.03	1.8	70	0
YUSM1009a	21 ± 4	ND	10 ± 1	1.2 ± 0.02	3.0 ± 0.04	1.5	75	0
TMB3102	42 ± 4	11.6 ± 1.1	13 ± 1	1.6 ± 0.02	3.3 ± 0.03	7.5	88	0.28 ± 0.02
TMB3104	20 ± 5	ND	6.1 ± 1	0.68 ± 0.01	2.6 ± 0.03	1.3	54	0

[a] Values are millimoles of C consumed or produced after 70 h, and they represent average values of fermentation based on triplicate experiments.
[b] 1 mmol of C from CO_2 is assumed to be formed for every 2 mmol of C from ethanol and acetic acid.
[c] ND, not detected.

supernatant was stored at −20°C until analyzed for protein concentration and enzyme activities. Protein concentrations were measured according to the method of Bradford (Bio-Rad, Rockford, Ill.) (4) with bovine serum albumin as the standard.

Enzyme assays. Enzyme activities were measured with a U-2000 model spectrophotometer (Hitachi Ltd., Tokyo, Japan). In all assays, the decrease in NAD(P)H was monitored at 340 nm at 30°C. The activities were expressed in units per milligram of protein; 1 U is equivalent to the amount of enzyme required to reduce 1 μmol of substrate/min.

AR activity was determined as previously described (17) in a total volume of 1 ml.

XI activity was assayed in two steps (5). This assay is different from the one used previously (37), with the following modifications: crude cell extract was mixed in 700 mM xylose, 10 mM $MnCl_2$, and 100 mM triethanolamine in a total volume of 500 μl. XI in the crude extract was allowed to convert xylose to xylulose for 10 min at 60°C. The reaction was stopped with 50% trichloric acid and subsequently neutralized with 2 M Na_2CO_3. In the second step, the decrease of NADH was measured when xylulose was converted to xylitol by sorbitol dehydrogenase (EC 1.1.1.14) in a mixture of neutralized sample, 10 μM NADH, 0.03 U of sorbitol dehydrogenase (Sigma-Aldrich Sweden AB), and 100 mM triethanolamine at 30°C and pH 7.0. The reaction was started by adding sorbitol dehydrogenase. The final assay volume was 1 ml. Standard curves were obtained with known concentrations of xylulose prepared as previously described (20).

XK activity measurements were based upon the method of Shamanna and Sanderson (25) and modified as described previously (9).

Fermentation conditions. Fermentation was carried out in 25-ml closed bottles filled with 25 ml of defined mineral medium (34) containing citrate buffer (pH 5.5), amino acids, and xylose or xylose plus glucose. The bottles were plugged with rubber stoppers, and a gas outlet was secured by inserting a cannula. Fermentation was performed at 30°C with agitation by magnetic stirring. The initial cell mass concentration was 10 g (dry weight)/liter. Xylose fermentation was carried out in triplicate, and xylose plus glucose fermentation was carried out in duplicate.

Oxygen-supplemented fermentation was carried out in 1-liter baffled flasks filled with 500 ml of medium (27) containing citrate buffer (pH 5.5), 50 g of xylose/liter, and amino acids. The initial cell mass concentration was 2 g (dry weight)/liter. Fermentation was carried out in duplicate.

Analytical methods. Concentrations of sugar substrates and fermentation products were determined using high-performance liquid chromatography (Beckman Instruments AB, Bromma, Sweden) with a hydrogen column (Aminex HPX-87H; Bio-Rad, Richmond, Calif.) at 45°C with 5 mM H_2SO_4 as the mobile phase with a flow rate of 0.6 ml min^{-1}. The compounds were detected with a refractive-index detector (RID 6A; Shimadzu, Kyoto, Japan).

The dry weight of cells was determined by filtering a known volume of culture broth through a predried 0.45-μm-pore-size nitrocellulose filter (Gelman Sciences, Ann Arbor, Mich.). After washing with 3 volumes of double-distilled water (Millipore, Bedford, Mass.) and drying in a microwave oven (Whirlpool, Benton Harbor, Mich.) for 8 min at a level of 120 W, the filter was weighed. The dry weight of the cells was determined in duplicate.

Calculations. Carbon balances, yields, and product formation were calculated using single carbon unit equivalents (moles of carbon) to permit comparison of hexose and pentose fermentation data (7). The theoretical yield of ethanol from xylose or glucose is 0.67 mol of C from ethanol/mol of C from xylose or glucose, which is equivalent to 0.51 g of ethanol/g of xylose or glucose. Yields of ethanol were calculated for total consumed sugars as well as for xylose. When calculating the ethanol yield from xylose with glucose as the cosubstrate, the theoretical amount of ethanol produced from glucose was subtracted from the total amount of ethanol. The carbon balance was calculated assuming that 1 mmol of C from CO_2 was formed for every 2 mmol of C from ethanol and acetic acid.

RESULTS

Construction of Δ*gre3* strain expressing *xylA* and *XKS1*. The gene *GRE3* was deleted from *S. cerevisiae* chromosome VIII. This recombinant *S. cerevisiae* strain was transformed with plasmids containing the *xylA* and *XKS1* genes encoding XI and XK. The reference strain was transformed with the same plasmids.

The promoter and terminator sequences of the *GRE3* gene were amplified by PCR (Fig. 2A). Plasmids pBR322, pGEM-T easy vector, pBluescript SK(−), pUSM1002 to -1005, and pUSM1007 and -1008 (Table 1) were used to generate pUSM1006 (Fig. 2A). pUSM1006 was cut with *Xba*I to linearize the fragment. The fragment was transformed into *S. cerevisiae* CEN.PK2-1C (Fig. 2B). Transformants with the plasmid cassette integrated into the promoter region of the *GRE3* gene (YUSM1006a) were selected on $SC^{-uracil}$ agar plates. The transformants were then grown in yeast extract-peptone-dextrose medium to allow homologous recombination between terminator regions and loss of the *URA3* marker gene and the *GRE3* gene. Replica plating with SC plates containing 5-FOA screened for transformants that had lost the *URA3* marker was performed. Transformants were generated by recombination in two ways. First, if the promoter regions combined, the original strain was obtained. Second, if the terminator regions combined, the whole plasmid cassette was lost as well as the *GRE3* gene (Fig. 2B). Deletion of the AR gene was confirmed by Southern blot analysis (data not shown). Strain CEN.PK2-1C from which the *GRE3* gene had been deleted was called YUSM1009a. *S. cerevisiae* CEN.PK2-1C and YUSM1009a were transformed with the replicative plasmid pBXI (37), resulting in TMB3101 and TMB3102, respectively. These two recombinant *S. cerevisiae* strains were subsequently transformed with the replicative plasmid pXks (9), resulting in TMB3103 and TMB1004, respectively.

Enzyme activities. Stationary phase cells were harvested, and the specific AR, XI, and XK activities of CEN.PK2-1C, YUSM1009a, and TMB3101-4 were measured (Table 2). Cells were harvested in the stationary phase to mimic fermentation, which was carried out with nongrowing cells. AR activity was present in CEN.PK2-1C, TMB3101 (reference strain, XI), and

TABLE 4. Carbon balances after fermentation of 50 g of xylose/liter and 20 g of glucose/liter[a]

Strain	Amt of C (mmol) (mean ± SD) from[b]:						Carbon recovery (%)	$Yield_{ethanol/xylose\ plus\ glucose}$[c]	$Yield_{ethanol/xylose}$[d]	Amt of ethanol (g/liter)
	Glucose	Xylose	Ethanol	Xylitol	Glycerol	Acetic acid	CO_2[e]			
CEN.PK2-1C	833 ± 18	46 ± 6	412 ± 30	46 ± 6	71 ± 6	20 ± 3	216	87	0.47 ± 0.02	0	9.5 ± 0.7
TMB3101	640 ± 16	63 ± 7	323 ± 5	39 ± 3	64 ± 3	16 ± 3	170	87	0.46 ± 0.02	0	7.4 ± 0.1
TMB3103	703 ± 14	84 ± 4	359 ± 11	59 ± 3	63 ± 3	24 ± 5	192	89	0.46 ± 0.03	0	8.3 ± 0.3
YUSM1009a	767 ± 17	58 ± 8	382 ± 5	13 ± 2	69 ± 2	18 ± 2	200	83	0.46 ± 0.02	0	8.8 ± 0.2
TMB3102	700 ± 15	192 ± 18	402 ± 19	17 ± 5	56 ± 6	41 ± 15	221	83	0.45 ± 0.03	0	9.2 ± 0.5
TMB3104	707 ± 14	502 ± 25	590 ± 24	30 ± 6	97 ± 3	114 ± 23	352	98	0.49 ± 0.02	0.23 ± 0.02	11.5 ± 0.6

[a] Displayed values are millimoles of C consumed or produced at the maximum ethanol point, and they represent the average values of fermentation based on duplicate experiments.
[b] Glucose and xylose were the substrates in the fermentation. Ethanol, xylitol, glycerol, acetic acid, and CO_2 were the products of fermentation.
[c] Results are millimoles of C from ethanol per millimole of C from xylose plus glucose. Results are means ± standard deviations.
[d] Results are millimoles of C from ethanol per millimole of C from consumed xylose. Ethanol yield on xylose was estimated by subtracting the theoretical ethanol amount (0.67 mmol of C/mmol of C from glucose consumed) formed from glucose from the total amount of ethanol and dividing by the amount of consumed xylose. Results are means ± standard deviations.
[e] 1 mmol of C from CO_2 is assumed to be formed for every 2 mmol of C from ethanol and acetic acid.

TMB3103 (reference strain, XI plus XK), whereas no AR activity was detected when the *GRE3* gene was deleted from YUSM1009a (Δ*gre3*), TMB3102 (Δ*gre3*, XI) and TMB3104 (Δ*gre3*, XI plus XK). The XI activity was similar with or without the *GRE3* gene. In TMB3101 and TMB3103, the specific XI activities were 0.90 and 0.55 U mg^{-1}, respectively. In the *GRE3*-deletion strains TMB3102 and TMB3104, the specific XI activities were 1.0 and 0.42 U mg^{-1}, respectively. XK activity was not detected in the reference strain CEN.PK2-1C, YUSM1009a, or TMB3101. In TMB3103 and TMB3104, the specific XK activities were 14 and 11 U mg^{-1}, respectively.

Substrate consumption and product formation in xylose fermentation. Six strains producing different levels of AR, XI, and XK were compared in batch fermentation (Table 3). None of the recombinant strains grew on xylose. Fermentation was performed under anaerobic conditions with 50 g of xylose/liter as the sole carbon source. For all six strains, the rate of xylose consumption was low, about 0.03 to 0.06 mmol of C/g of cells/h. Ethanol was produced only by TMB3102 (Δ*gre3*, XI), in the amount of 11.6 mmol of C with a yield of 0.28 mmol of C from ethanol/mmol of C from consumed xylose. In strains from which the *GRE3* gene was deleted, the xylitol production decreased by half. The acetic acid yield was similar in all strains and the glycerol yield increased twofold in Δ*gre3* strains. Overall carbon balances were calculated, and the consumed carbon was only recovered to 54 to 88%. No other products were detected by high-performance liquid chromatography.

Influence of glucose on anaerobic xylose fermentation. Based on the results obtained from the xylose fermentation, the influence of glucose on xylose consumption and product formation was investigated next. The product formation during anaerobic batch fermentation with 50 g of xylose/liter was compared to that with 20 g of glucose/liter (Table 4). The rate of xylose consumption in these fermentations was higher than in those with xylose as the sole carbon source. This supports previous observations that a cosubstrate increases the uptake rate of xylose (18, 31, 33). The xylose uptake was between 1.5 and 2.8 mmol of C/g of cells/h for CEN.PK2-1C, TMB3101 (reference strain, XI), TMB3103 (reference strain, XI plus XK), and YUSM1009a (Δ*gre3*). In TMB3102 (Δ*gre3*, XI) and TMB3104 (Δ*gre3*, XI plus XK), the xylose uptake increased to 6.4 and 10 mmol of C/g of cells/h, respectively. In Δ*gre3* strains, xylitol formation decreased two- to threefold. The xylitol yields on consumed xylose were low in TMB3102 and TMB3104: 0.09 and 0.06 mmol of C from xylitol/mmol of C from consumed xylose, respectively. Glycerol formation and acetate formation were similar for all strains except TMB3104, for which formation of both acetate and glycerol was higher. The ethanol yield on xylose was 0.23 mmol of C from ethanol/mmol of C from xylose in TMB3104. When calculating the ethanol yield from xylose, the theoretical amount of ethanol produced from glucose was subtracted from the total amount of ethanol. With this reasoning, ethanol formation from xylose was formed only in TMB3104. In TMB3102 the increased xylose uptake did not significantly decrease the ethanol yield from total sugars or increase the xylitol formed, suggesting that ethanol was also formed from xylose. Overall carbon balances were calculated, and the consumed carbon was recovered to 83 to 98%.

Influence of oxygen on xylose fermentation. Oxygenation of xylose fermentation did not improve xylose consumption, and

ethanol formation was not detected. After 70 h of fermentation, 4.9 ± 1.0 mmol of C from xylitol was produced by CEN.PK2-1C, TMB3101 (reference strain, XI), and TMB3103 (reference strain, XI plus XK), and 2.3 ± 0.2 mmol of C from xylitol was produced by YUSM1009a (Δ*gre3*), TMB3102 (Δ*gre3*, XI), and TMB3104 (Δ*gre3*, XI plus XK). Small amounts of other products were also formed, about 0.9 mmol of C from glycerol for all six strains and 1.3 mmol of C from acetate for CEN.PK2-1C, TMB3101, and TMB3103. No acetate was detected in the Δ*gre3* strains.

DISCUSSION

Deletion of the *GRE3* gene in *S. cerevisiae* decreased xylitol formation two- to threefold but not completely. During xylose fermentation, xylitol may also be formed from xylulose by an endogenous XDH (21). The equilibrium of this reaction favors xylitol formation. Xylitol could also be formed by putative ARs that have been identified in *S. cerevisiae* (11), although no AR activity was detected in the Δ*gre3* strains in this study.

So far only XI from *T. thermophilus* has been actively produced in *S. cerevisiae* (37). Ethanol formation from xylose was shown by this recombinant *S. cerevisiae*, whereas ethanol formation was not detected when TMB3101 (reference strain, XI) assimilated xylose. The difference is attributed to different fermentation temperatures (30°C compared to 38°C), host strains, and fermentation media (defined medium compared to SC medium). The XI activity is about twice as high at 38°C than at 30°C (37). *S. cerevisiae* H158 was used as the host strain in the previous study for heterologous XI production (37). This strain forms ethanol with a higher yield from xylose than the currently used CEN.PK strain when transformed with genes for xylose-metabolizing enzymes (16).

Xylitol inhibits the enzymatic isomerization of xylose to xylulose by XI (39), and less xylitol was produced in TMB3102 than in TMB3101 (reference strain, XI) (Fig. 1). Ethanol formation from xylose in Δ*gre3* strains TMB3102 (in xylose media) and TMB3104 (in xylose plus glucose media) was also favored by a reduced loss of carbon from xylitol, so the carbon flow was redirected towards the pentose phosphate pathway and the central metabolism.

TMB3104 produced no ethanol in xylose media. In this strain XK was overproduced. Metabolic modeling has suggested that a constitutively overproduced kinase in the beginning of a metabolic pathway depletes the intracellular ATP pool and causes intracellular accumulation of sugar phosphates (30). However, intracellular concentrations of ATP, xylulose, and xylulose-5-phosphate in strains overproducing XK did not differ from those in strains not overproducing XK (data not shown). On the contrary, reduced ATP levels were observed in a recombinant *S. cerevisiae* producing XR and XDH from *P. stipitis* and endogenous XK (32). Thus, an optimal level of XK seems to be important for efficient xylose fermentation.

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