

Molecular Cloning of *XYL3* (D-Xylulokinase) from *Pichia stipitis* and Characterization of Its Physiological Function

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***XYL3*, which encodes a D-xylulokinase (EC 2.7.1.17), was isolated from *Pichia stipitis* CBS 6054 genomic DNA by using primers designed against conserved motifs. Disruption of *XYL3* eliminated D-xylulokinase activity, but D-ribulokinase activity was still present. Southern analysis of *P. stipitis* genomic DNA with *XYL3* as a probe confirmed the disruption and did not reveal additional related genes. Disruption of *XYL3* stopped ethanol production from xylose, but the resulting mutant still assimilated xylose slowly and formed xylitol and arabinitol. These results indicate that *XYL3* is critical for ethanol production from xylose but that *P. stipitis* has another pathway for xylose assimilation. Expression of *XYL3* using its *P. stipitis* promoter increased *Saccharomyces cerevisiae* D-xylulose consumption threefold and enabled the transformants to produce ethanol from a mixture of xylose and xylulose, whereas the parental strain only accumulated xylitol. In vitro, D-xylulokinase activity in recombinant *S. cerevisiae* was sixfold higher with a multicopy than with a single-copy *XYL3* plasmid, but ethanol production decreased with increased copy number. These results confirmed the function of *XYL3* in *S. cerevisiae*.**

D-Xylulose is phosphorylated to D-xylulose-5-phosphate by D-xylulokinase (EC 2.7.1.17) before it enters glycolysis through the nonoxidative pentose phosphate pathway. D-Xylulokinase is the first step in the metabolism of xylose that is common to both bacteria and eukarya (14, 20, 21).

The bacterial gene for D-xylulokinase (*xylB*) was first cloned from *Escherichia coli* in 1984 (26, 34). In 1989 Ho and Chang (19) reported cloning the *Saccharomyces cerevisiae* gene for D-xylulokinase by complementing an *Escherichia coli xylB* deficiency. It was also sequenced as a part of the *S. cerevisiae* genome project and named *XKS1* (32). Of the few putative eukaryotic genes for D-xylulokinases, only the *S. cerevisiae* (31) and the presently reported *Pichia stipitis* genes have been characterized (45). The present report is the first physiological characterization of a D-xylulokinase-encoding gene from a eukaryote that uses xylose for growth and ethanol production.

Among the native xylose-fermenting yeasts, *Pachysolen tannophilus*, *Candida shehatae*, and *Pichia stipitis* can ferment xylose to ethanol with high yields (8, 9, 43). However, relative to glucose fermentation by *Saccharomyces*, these xylose-fermenting yeasts display lower ethanol production rates. Moreover, yeast xylose fermentations require low levels of aeration for optimal ethanol production (10, 15). In an attempt to overcome these problems, researchers have cloned and expressed the genes for xylose reductase (XR) and xylitol dehydrogenase (XDH) (*XYL1* and *XYL2*, respectively) from *P. stipitis* in *S. cerevisiae* (2, 25, 47, 49). The resulting transformants can grow

on xylose aerobically and produce ethanol under oxygen-limited conditions in low yield (23, 46).

The *S. cerevisiae* xylulokinase gene, which corresponds to *XKS1* or *YGR194C* (32), increased ethanol and decreased xylitol production in a *Saccharomyces* sp. that had been engineered with *P. stipitis XYL1* and *XYL2* (17, 18). Richard et al. (31), along with Stevis and Ho (44), showed that *XKS1* is the only route for xylulose metabolism in this yeast. Since the first report by Chang and Ho (4), others have overexpressed *Saccharomyces XKS1* along with the *P. stipitis* genes for XR and XDH in *S. cerevisiae* (12, 17, 48). These efforts have enabled higher ethanol yields from D-xylose in recombinant *Saccharomyces* strains, but these heterologous transformants generally do not perform as well as native *P. stipitis* or *C. shehatae* (10).

It is not clear that overexpression of *XKS1* is entirely beneficial. In fact, overexpression of *XKS1* in a different *Saccharomyces* genetic background was reported to inhibit growth on D-xylulose (32). Other studies by Toivari et al. (48) and Richard et al. (31) did not confirm this finding, but Johansson et al. (24) found that overexpression of *S. cerevisiae XKS1* reduced xylose consumption by 50 to 80% in *S. cerevisiae* transformants even while it increased the yield of ethanol from xylose, and they cautioned against unmodulated overexpression of this gene. Thus, some controversy surrounds this approach. The *S. cerevisiae* D-xylulokinase is also active on D-ribulose (31), whereas the *P. stipitis* D-xylulokinase is not (13).

The objective of our work was to clone and characterize the *P. stipitis* xylulokinase gene (*XYL3*) so that it might be used for engineering xylulose metabolism without the side activity seen with *S. cerevisiae XKS1* (51).

MATERIALS AND METHODS

Strains. Microbial strains used in this study are listed in Table 1. *P. stipitis* CBS 6054 was the source of all sequenced DNA. Michael Culbertson (Department of Genetics, University of Wisconsin) kindly provided *S. cerevisiae* 679 (*MAT α trp1-2*). *Escherichia coli* XL-1 Blue MRF and SOLR (Stratagene, La Jolla, Calif.)

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>P. stipitis</i> CBS 6054	Wild type	CBS ^a
<i>P. stipitis</i> UC7	<i>ura3-2</i>	27
<i>P. stipitis</i> YS30	Isogenic to <i>P. stipitis</i> UC7 except <i>xy13::URA3</i>	This study
<i>S. cerevisiae</i> 679	<i>MATα trp1-289</i>	M. Culbertson
<i>S. cerevisiae</i> 679C	<i>S. cerevisiae</i> 679(pRS424)	This study
<i>S. cerevisiae</i> 679[pYS31N]	<i>S. cerevisiae</i> 679(pYS31N)	This study
<i>S. cerevisiae</i> 679[pYS32N]	<i>S. cerevisiae</i> 679(pYS32N)	This study
Plasmids		
pRS314	<i>TRP1, CEN/ARS</i>	42
pRS424	<i>TRP1, 2μm origin</i>	6
pX3	<i>XYL3</i> in pBluescript KS(+)	This study
pYS03	<i>XYL3</i> disruption cassette containing <i>xy13::URA3</i>	This study
pYS31N	<i>XYL3</i> in pRS314	This study
pYS32N	<i>XYL3</i> in pRS424	This study

^a CBS, Centraalbureau voor Schimmelcultures (Delft, The Netherlands).

were used in conjunction with the λ -ZAPII genomic DNA library of *P. stipitis* (28).

Media and culture conditions. *E. coli* was grown in Luria-Bertani (LB) medium. Ampicillin (50 μ g/ml) was added to the medium when required. Yeast strains were grown in yeast peptone (YP) medium (10 g of yeast extract and 20 g of Bacto peptone per liter) or yeast synthetic complete (YSC) medium containing 6.7 g of yeast nitrogen base (YNB) without amino acids (Difco, Detroit, Mich.) per liter, which was complemented with appropriate amino acids and nucleotides according to Rose et al. (33). Glucose (20 g/liter) or xylose (40 g/liter) was used as a carbon source. For xylulose fermentation, a 20:80 xylulose-xylose mixture was prepared using xylose isomerase (Novo Nordisk, Copenhagen, Denmark). Yeast cells were cultivated at 30°C in 50 ml of medium in a 125-ml Erlenmeyer flask, and oxygen transfer rates were determined by sulfite oxidation (7).

Enzymes, primers, and chemicals. Restriction enzymes, DNA-modifying enzymes, and other molecular reagents were obtained from New England Biolabs (Beverly, Mass.), Promega (Madison, Wis.), Stratagene (La Jolla, Calif.), and Roche Biochemical (Indianapolis, Ind.). Reaction conditions were as recommended by the suppliers. All general chemicals were purchased from Sigma (St. Louis, Mo.). Primers for PCR and sequencing were synthesized by Sigma-Genosys (The Woodlands, Tex.).

Yeast transformation. A yeast EZ-Transformation kit (Bio 101, Carlsbad, Calif.) was used for all yeast transformations according to the manufacturer's instructions. Transformants were selected on YSC medium containing 20 g of glucose per liter. Necessary amino acids were added if required.

Cloning of *P. stipitis* xylulokinase gene. Homologous segments of protein sequences from bacterial D-xylulokinases, along with other putative eukaryotic proteins having similarity to that coded by the *S. cerevisiae* YGR194C open reading frame, were aligned against the *S. cerevisiae* protein sequence to identify the highly conserved regions at the N terminus, LGFDLSTQQ and QQHGS VYWS. Primers were designed against these motifs based on a *P. stipitis* codon usage table (39). A 300-bp fragment was amplified by *Pfu* polymerase (Stratagene, La Jolla, Calif.) from the genomic DNA of *P. stipitis* CBS 6054 using primers 1 (5'-CTATCTTGGGTTTGATCTTTTCGACC-3') and 2 (5'-GACCAG TAGACAGACCGTGCTGCT-3'). The amplicon was used as a probe to screen a λ -ZAPII *P. stipitis* genomic DNA library (28). A total of 3×10^6 plaques were initially screened. After the tertiary screening, one plaque remained positive. The single-stranded DNA was converted to double-stranded DNA in a phagemid, pBluescript vector as described by Lu et al. (28).

DNA sequencing. *XYL3* was sequenced by using an Applied Biosystems model 371 DNA sequencer (Perkin-Elmer). Sequence analysis was performed using Genetics Computer Group sequence analysis (GCG, Madison, Wis.) and DNAMAN software (Lynnon BioSoft, Vaudreuil, Quebec, Canada).

Multiple alignment and phylogenetic analysis of protein sequence. Homologues of *P. stipitis* xylulokinase were identified using Blast search program at the NCBI web site (<http://www.ncbi.nlm.nih.gov/BLAST/>). They were then aligned using ClustalW with default settings at the EBI website (<http://www.ebi.ac.uk/clustalw>). A phylogenetic tree was generated from the alignments using the neighbor-joining method (36). By throwing out the sequences in the gap, am-

biguous parts of the alignment were excluded from the phylogenetic analysis. For bootstrap analysis, a multiple alignment was sampled 10,000 times. Bootstrap values are expressed in percentages and placed at nodes.

Plasmid construction. Plasmids used in this study are summarized in Table 1. Plasmid DNA was isolated by QIAprep spin plasmid kit (Qiagen Inc., Chatsworth, Calif.). We isolated yeast genomic DNA using a genome DNA isolation kit (Bio 101, Vista, Calif.). *PstI* digestion of pX3 produced a 2.9-kbp fragment containing the *XYL3* gene with its native promoter. A single-copy vector (pYS31N) was constructed by inserting the 2.9-kbp *PstI*-*PstI* fragment of *XYL3* into the *PstI* site of pRS314 (42). Plasmid pRS32N (multicopy vector) was made by inserting the same 2.9-kbp fragment into the *PstI* site of pRS424 (6).

The disruption cassette, pYS03, was constructed by inserting a *Bam*HI-*Bam*HI fragment carrying *P. stipitis* *URA3* into the *Bgl*II site located in the middle of the *XYL3* open reading frame in pYS31N. The 1.2-kbp *Bam*HI-*Bam*HI fragment carrying *URA3* was amplified from *P. stipitis* genomic DNA by PCR using primers 3 (5'-CGGGATCCGCTTATGTTGCATCTCAAA-3') and 4 (5'-CGGGATCCCGAATAGGCCTCTGCTGT-3'). The 2.9-kbp fragment containing the disruption cassette was amplified from pYS31N by PCR with primers 5 (5'-GT TGACTCACAATGACC-3') and 6 (5'-CCGATCCATTCCTTCTTGG-3').

PCR screening of *XYL3* disruptants. Putative *XYL3* disruption mutants were screened by colony PCR. Yeast colonies were lysed at 37°C for 1 h using the whole cell yeast PCR kit (Bio 101, Vista, Calif.). Primers 5 and 6 were used in the PCR screening. The resulting lysate was mixed with 200 μ M deoxynucleoside triphosphates, 10 μ M primers, and 1.5 mM MgCl₂ to a final volume of 100 μ l; then, the mixture was incubated at 95°C for 15 min. Five units of *Taq* DNA polymerase (Roche Biochemicals, Indianapolis, Ind.) was added to the mixture, and the reaction was performed at 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 40 cycles, followed by 72°C for 4 min. The product was stored at 4°C.

Southern blot hybridization. Genomic DNA preparations digested with restriction enzymes were electrophoresed in 8 g of agarose per liter. A Southern transfer was then performed by capillary action according to the method of Sambrook et al. (37). Randomly primed DNA labeling with the purified *XYL3* coding region as a template generated a probe labeled with digoxigenin-11-dUTP (Roche Biochemicals, Indianapolis, Ind.). DIG Easy Hyb solution (Roche, Indianapolis, Ind.) was used for hybridization at 37°C.

Preparation of crude extracts. *S. cerevisiae* and *P. stipitis* were grown to exponential phase in YSC medium supplemented with appropriate amino acids and nucleotides and harvested by centrifugation. The pellet was washed and suspended in the buffer (100 mM phosphate buffer, 1 mM EDTA, 5 mM β -mercaptoethanol, pH 7.0). The suspended cells were mixed with glass beads (Sigma, St. Louis, Mo.), vortexed at the maximum rate in bursts of 30 to 120 s, and then cooled on ice for a similar period. This procedure was repeated for up to 8 min of vortexing or until microscopic examination showed >80% cell breakage. The crude extract, collected after centrifugation for 10 min at 15,000 \times g, was used in the enzyme assay.

Xylulokinase and ribulokinase assays. Xylulokinase and ribulokinase activities were measured according to the method of Shamanna and Sanderson (38). For the *P. stipitis* xylulokinase assay, another blank without pyruvate kinase and lactate dehydrogenase was used to minimize the interference of xylitol dehydrogenase activity in *P. stipitis*. We used a photodiode array spectrophotometer (Hewlett Packard, Wilmington, Del.) to monitor the reaction by absorbance at 340 nm. All assays were performed within 2 to 4 h of cell breakage. Control homogenates with protease inhibitor cocktail (Sigma, St. Louis, Mo.) did not show significant differences in activity. Our assay could detect 0.001 U/mg of protein. One unit of activity is defined as the amount of enzyme that phosphorylates 1 μ mol of xylulose per min at 30°C. Protein concentration was determined by the BCA method (Pierce, Rockford, Ill.).

Analytical methods. Glucose, xylose, xylitol, xylulose, and ethanol concentrations were determined by high-pressure liquid chromatography (HPLC) (HP, Wilmington, Del.) with an Ion 300 column (Interaction Chromatography, San Jose, Calif.). The variance was approximately 5% in replicate samples. For the gas chromatography-mass spectrometry (GC-MS) analysis, fermentation samples were concentrated by rotary evaporation, dissolved with pyridine, and converted into a trimethylsilyl derivative. Gas chromatography (Finnigan, Austin, Tex.) with a ZB-5-MS column (Phenomenex) was used with the following conditions: 100°C for 1 min, then increasing to 300°C at 20°C/min. Cell growth was monitored by optical density at 600 nm (OD₆₀₀). One OD₆₀₀ unit was equivalent to 0.17 g of cells/liter for *S. cerevisiae* and 0.21 g of cells/liter for *P. stipitis*.

Nucleotide sequence accession numbers. The sequences of the coding region and 5'- and 3'-flanking regions were deposited in GenBank (AF127802 and AAF72328).

<i>Bacillus subtilis</i>	-----MKYVIGIDLGTSAVKTILVN-QNGKVAETS	30
<i>Haemophilus influenzae</i>	-----MYIGIDCGTQGTAKAIVLDSVQKKVIGVGY	29
<i>Klebsiella aerogenes</i>	-----MYIGIDLGTSGVKAILLN-EQGEVVASHT	28
<i>Lactobacillus pentosus</i>	-----MSAVVLGIDLGTSAVKVSAID-KQGNVVAQAS	31
<i>Staphylococcus xylosus</i>	-----MAYVIGIDIGTSAKTLVVN-KSGDVVESYS	30
<i>Streptomyces rubiginosus</i>	-----MSAAEGPLVVGVDSTQSTKALVVDVATGRVVASGQ	36
<i>Caenorhabditis elegans</i>	-----MKDGFGLIDLSTQQIKAVIID-QNGKVVHTTA	31
<i>Arabidopsis thaliana</i>	-----MADLSLPPDLSLFLGFDSSSTQSMKATVLD-SNLNLIKTEL	38
<i>Saccharomyces cerevisiae</i>	MLCSVIQRQTRVSNMTMSLDSYYLGFDLSTQQLKCLAIN-QDLKIVHSET	49
Sequence used for primer design	<u>LGFDLSTQQLK</u>	
<i>Pichia stipitis</i>	-----MTTTPFDAPDKLFLGFDLSTQQLKIVTD-ENLAALKTYN	39
<i>Bacillus subtilis</i>	S----NVQAKDIDIGISYSGQMHLVLLDQDRQ-----	92
<i>Haemophilus influenzae</i>	AKNSPHFSPNLVKGIISGQQHGLVMLDKNDR-----	95
<i>Klebsiella aerogenes</i>	-----HDSLRHVKGLGIAGQMHGATLLDKSLQ-----	88
<i>Lactobacillus pentosus</i>	S----EVTADQIEGLSYSGQMHLVLLDESAT-----	93
<i>Staphylococcus xylosus</i>	-----HYTHNDLTGIFSFGQMHLVVIDQEGN-----	90
<i>Streptomyces rubiginosus</i>	A-----HEAAAVSIGGQQHGLVTLDGHGEP-----	93
<i>Caenorhabditis elegans</i>	N----GWT-DKLRGISGCAQQHGTIVYWKNGAENS LKGLDESRS-----LAEQ	118
<i>Arabidopsis thaliana</i>	A----NFDFAKVI AVSGSQQHGVS VYWSKGSSEVLRSLDSKRS-----LKEQ	127
<i>Saccharomyces cerevisiae</i>	A----KFPPLNKVM AVSGSQQHGVS VYWSQAESLLEQLNKKPEKDLLHYV	138
Sequence used for primer design	<u>SGSCQQHGSVYWS</u>	
<i>Pichia stipitis</i>	D----GFPFNKVVIGISGSCQQHGSVYWSRTAEKVLSELDAESS-----LSSQ	128

FIG. 1. Alignment of xylulokinase sequences from several bacteria and eukaryotes against the *P. stipitis* *XYL3* sequence. Highly conserved sequences are in bold. Sequences used for primer design are underlined.

RESULTS

Cloning of *XYL3* from *P. stipitis*. To clone *XYL3*, we identified two highly conserved regions at the N terminus, LGFDLSTQQLK and SGSCQQHGSVYWS, by aligning ungapped Blast segments (1) of protein sequences from bacterial xylulokinases reported in the literature and putative D-xylulokinases from unidentified open reading frames of eukaryotic proteins (*Caenorhabditis elegans* [accession no. YNE7_CAEEL] and *Arabidopsis thaliana* [BAA97229]) against the *S. cerevisiae* sequence from the *S. cerevisiae* sequencing project, *YGR194C* (Fig. 1). LGFDLSTQQLK is involved in phosphate binding, which is a conserved region common to sugar kinases (3). The function of the second region is not known.

Using primers designed to these two regions, we successfully amplified a 300-bp fragment from *P. stipitis* genomic DNA. One open reading frame of the nucleotide sequence of this fragment showed homology to previously cloned xylulokinase genes. This fragment was then used as a probe to screen phage plaques from the λ -ZAPII genome library of *P. stipitis* CBS 6054. After three rounds of screenings, a positive clone was identified and confirmed by Southern blots. This 5.3-kbp clone was named pX3.

***XYL3* sequence analysis.** The *XYL3* sequence (GenBank accession no. AF127802 and AAF72328) from pX3 contained an open reading frame of 1,872 nucleotides, coding for a polypeptide of 623 amino acids with a total calculated molecular mass of 69,198 Da, which agreed well with the previous result (71 kDa) from a *P. stipitis* xylulokinase purification study (13). Sequence analysis of the 900 bp 5' to the open reading frame identified three putative TATA elements located at -180 to -188, -286 to -294, and -335 to -342.

A phylogenetic analysis showed that the *P. stipitis* D-xylulokinase protein had diverged significantly from the 16 other known yeast, bacterial, plant, and animal xylulokinases (Fig. 2). The *XYL3* open reading frame was only 28% identical at the DNA level to its nearest neighbor, *S. cerevisiae* D-xylulokinase *XKS1* (*YGR194C*) (19, 32), while at the amino acid level, the *P. stipitis* xylulokinase displayed 44% identity to the *S. cerevi-*

siae homologue. The *P. stipitis* xylulokinase protein belongs to the FGGY, FGGY family of carbohydrate kinases (Prosite PDOC00408), which includes D-xylulokinase (EC 2.7.1.17), L-xylulokinase (EC2.7.1.53), glycerol kinase (EC 2.7.1.30), glucokinase (EC 2.7.1.12), and L-fuculokinase (EC 2.7.1.51) (30).

Disruption of *XYL3*. To investigate the function of the *XYL3* gene product, we disrupted *XYL3* in a *P. stipitis* strain, FPL-UC7 (*ura3*) (27), by gene replacement (35) with *P. stipitis* *URA3* in the middle of the *XYL3* coding region (Fig. 3A). *Ura*⁺ strains were selected on YSC-glucose without uracil and then replica plated on YSC with xylose medium to screen for

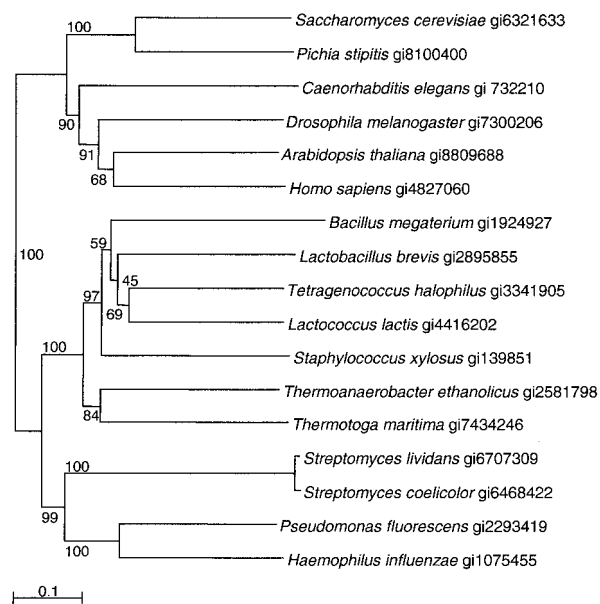


FIG. 2. Phylogenetic tree of *P. stipitis* xylulokinase protein homologues found in GenBank. Homologues of xylulokinase were identified by Blast search, and found protein sequences were then aligned. A phylogenetic tree was generated from the aligned regions after excluding sequences creating gaps.

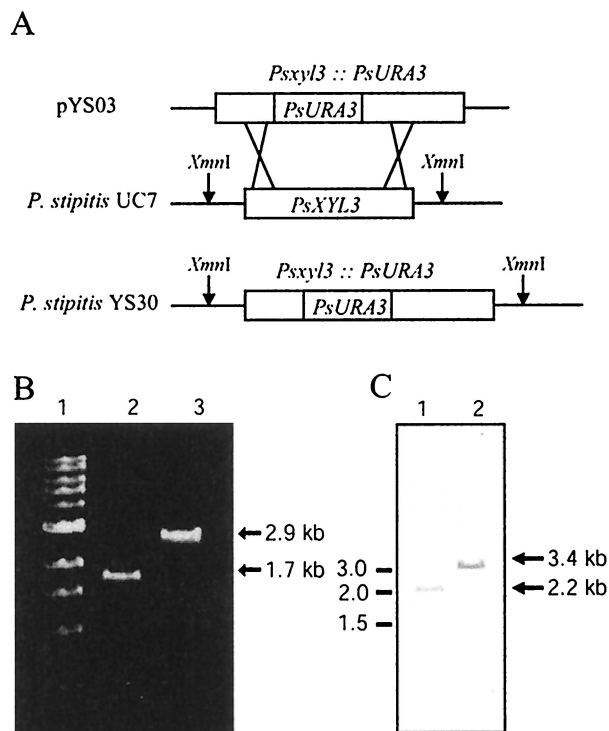


FIG. 3. Construction of *XYL3* gene disruption cassette and confirmation of *XYL3* disruption by PCR and Southern blotting. (A) Systematic representation of the disruption cassette (pYS03). The linear disruption cassette was amplified by PCR from pYS03. It was then introduced into the chromosome by gene replacement. The pYS03 contains 1.2 kbp of the *P. stipitis URA3* gene in the middle of the *XYL3* coding region. (B) PCR with specific primers for amplification of the 1.7-kbp *XYL3*. The templates were used as follows: lane 1, markers; lane 2, *P. stipitis* FPL-UC7 genomic DNA; lane 3, *P. stipitis* FPL-YS30 genomic DNA. (C) Southern blot analysis of *XYL3* disruption with the probe made from the *XYL3* coding region. The genomic DNAs from *P. stipitis* FPL-UC7 (lane 1) and *P. stipitis* YS30 (lane 2) were digested with *XmnI*. The parental strain, *P. stipitis* FPL-UC7, showed a 2.2-kbp band and the *XYL3* disruptant, *P. stipitis* FPL-YS30 (*xyl3::URA3*), showed a 3.4-kbp band. Positions of size markers are shown to the left.

colonies deficient in xylose assimilation. All *Ura*⁺ transformants could grow on YSC medium with xylose as a sole carbon source, but 2 of the 50 selected colonies grew very slowly.

Colony PCR and Southern blot analyses were used to determine whether these were *XYL3* disruptants. In colony PCR, the slow-growing transformants displayed a 2.9-kbp fragment corresponding to the *URA3* gene inserted into the coding region of *XYL3*. In contrast, the *Ura*⁺ colonies resulting from random integration of the disruption cassette or from reversion showed a 1.7-kbp fragment that corresponded to the coding region of *XYL3* (Fig. 3B). One of the *XYL3* disruptants was named FPL-YS30 (*xyl3::URA3*). Genomic DNA from the parental strain, FPL-UC7 (*ura3*), or the *XYL3* disruptant, FPL-YS30, was digested with *XmnI*. After probing the Southern blot with an *XYL3* fragment, FPL-YS30 displayed a 3.4-kbp band corresponding to insertion of the 1.2-kbp fragment of *URA3* into *XYL3*. However, the parental strain showed only a single 2.2-kbp band (Fig 3C). This confirmed the disruption and indicated that only a single isozyme of *XYL3* was present in the genome.

The frequency of site-specific integration of the disruption cassette into the *P. stipitis* chromosome was low. Only 4% of the *Ura*⁺ transformants were true disruptants and showed slower growth phenotypes on xylose. This frequency was in line with our previous disruption studies of the *P. stipitis ADH* (5) and *CYC1* (40) genes.

Growth experiments on various carbon sources. Because xylulokinase belongs to a family that includes glycerol kinase and because there are structural similarities between xylulose and fructose, we tested FPL-YS30 for growth on fructose or glycerol as a sole carbon source (Fig. 4). FPL-YS30 showed extremely slow growth on D-xylose, but growth on the other carbon sources was comparable to that of the parental strain.

***XYL3* disruption mutant FPL-YS30 (*xyl3::URA3*) accumulates polyols.** We could not detect any significant differences in growth, sugar consumption, or ethanol production between the parental strain and FPL-YS30 when glucose was used as the carbon source (data not shown). The main products from both strains were biomass and ethanol. In contrast, FPL-YS30 displayed significant differences from the parental strain, FPL-UC7, in xylose fermentation (Fig. 5A and B). FPL-UC7 consumed 42 g of xylose/liter within 24 h and produced 16 g of ethanol/liter, with a negligible amount of xylitol production. However, xylose assimilation in the disruptant FPL-YS30 was severely hindered (0.98 g of xylose per g of cells per h compared with 4.2 g of xylose per g of cells per h for FPL-UC7). Cells of *P. stipitis* FPL-YS30 required about 70 h to consume 43 g of xylose/liter. Specific growth rates of the disruptant and parental strains on xylose were 0.06 and 0.28 h⁻¹, respectively. In contrast to the parental strain, FPL-YS30 did not produce detectable amounts of ethanol from xylose, and it accumulated up to 13.2 g of xylitol per liter in the medium.

The *XYL3* disruptant also produced an unknown product that eluted adjacent to xylitol during HPLC analysis. GC-MS analysis and sample loading with standard compounds showed it to be arabinitol, although our analytical methods could not determine its stereochemistry. Thus, the *XYL3* disruptant produced both arabinitol and xylitol, whereas the parental strain

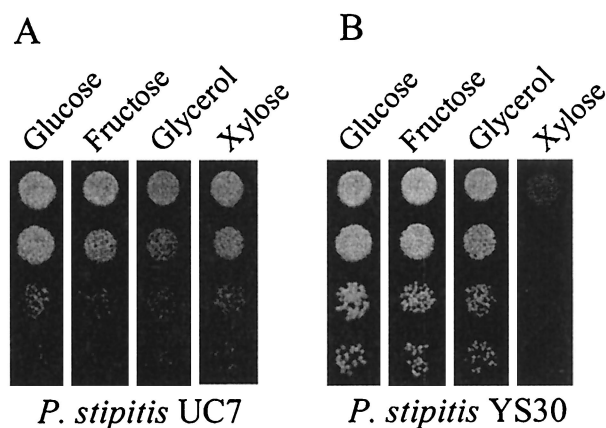


FIG. 4. Growth of the parental and *xyl3::URA3* disruptant strains on various carbon sources. (A) *P. stipitis* FPL-UC7 (*ura3*); (B) *P. stipitis* FPL-YS30 (*xyl3::URA3*). YP medium (10 g of yeast extract, 20 g of Bacto peptone, and 20 g of agar per liter) with 20 g of each carbon source per liter was used, and cells were serially diluted. Approximately 10⁴ cells were spotted in the undiluted (top) droplet.

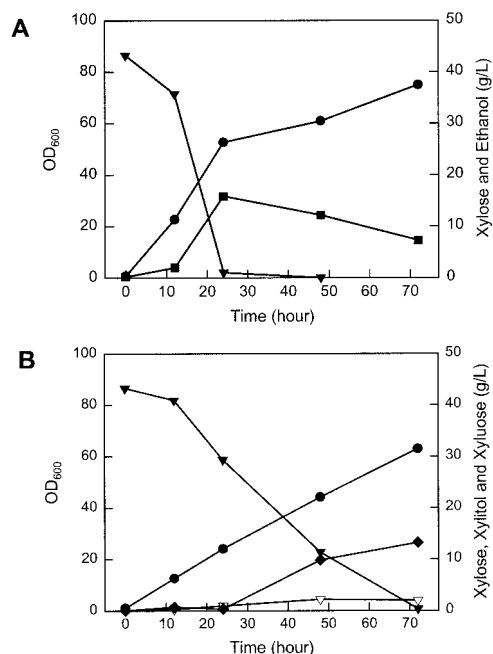


FIG. 5. Fermentation of xylose by *P. stipitis* FPL-UC7 (*ura3*) (A) and *P. stipitis* FPL-YS30 (*xyl3::URA3*) (B). Cells were cultured in YPX with 40 g of xylose per liter, with an oxygen transfer rate of 5.3 mM h⁻¹. Symbols: ▼, xylose; ▽, xylulose; ◆, xylitol; ●, cell density; ■, ethanol. Results in A and B are each from a single representative trial.

did not accumulate significant amounts of these two polyols. These results showed that the *XYL3* disruptant could grow on xylose, but the fate of consumed xylose is different in FPL-YS30 and its parent.

In vitro xylulokinase and ribulokinase assays of *P. stipitis* FPL-UC7 and *P. stipitis* FPL-YS30. *P. stipitis* FPL-YS30 could grow slowly on xylose. However, our enzyme assays from the culture of the *XYL3* disruptant could not detect D-xylulokinase activity. By contrast, a control culture of the parental strain, FPL-UC7, showed xylulokinase activity of 0.24 U/mg under the same conditions. Assays performed with and without protease inhibitor did not show significant differences in activity. The specific activity of D-ribulose was unaltered in the mutant and was approximately 0.07 U/mg of protein for FPL-UC7 and 0.11 U/mg of protein for FPL-YS30.

***XYL3* is actively expressed in *S. cerevisiae* under the native *XYL3* promoter.** We confirmed the function of the *XYL3* gene product by heterologous expression in *S. cerevisiae*. A 2.9-kbp *PstI-PstI* fragment containing 900 bp of 5' untranslated region and 200 bp of 3' untranslated region in addition to the D-xylulokinase coding region was subcloned into a CEN/ARS-based (pYS31N) or a 2 μ m-based vector (pYS32N). Both plasmids were introduced into *S. cerevisiae* 679, and transformants were selected on YSC-glucose medium without tryptophan. After growing the cells in YSC-glucose medium, we measured xylulokinase activities in the crude extracts from the 679 [pYS31N] centromeric and 679[pYS32N] 2 μ m-based strains as well as the parental strain. The 679[pYS31N] and 679[pYS32N] strains showed much higher xylulokinase activities (0.019 ± 0.002 U/mg and 0.121 ± 0.008 U/mg, respectively) than the parental strain, which did not have detectable activity.

We also observed that xylulokinase activity in the extract from the cells bearing the 2 μ m-based vector, 679[pYS32N], were sixfold higher than that from cells bearing the centromeric vector, 679[pYS31N].

Xylulose fermentation by recombinant *S. cerevisiae*. *S. cerevisiae* naturally ferments D-xylulose, a keto-isomer of xylose, but it uses xylulose at one-tenth the rate it uses glucose (52). Xylulose phosphorylation has been discussed as a possible rate-limiting step in xylose fermentation by recombinant *S. cerevisiae* (17). We tested whether *XYL3* expression can enhance xylulose fermentation by *S. cerevisiae* under respirofermentative conditions (oxygen transfer rate, 5.3 mM O₂/h). For the xylulose fermentation experiment, a mixture of xylose and xylulose was used.

S. cerevisiae 679[pYS31N] and 679[pYS32N] used the D-xylulose about three times faster than the parental strain transformed with the control vector, pRS424, which carries *TRP1* without the *XYL3* insert (Table 2). The parental strain consumed only a fraction of the available D-xylulose and produced no ethanol during entire fermentation trials (Fig. 6A), whereas both recombinant strains carrying *XYL3* completely utilized the added 20 g of D-xylulose per liter within 6 days and produced ethanol from D-xylulose (Fig. 6B and C). The maximum ethanol concentration observed was 4.0 g/liter in the transformant bearing the single-copy vector (679[pYS31N]) and 2.8 g/liter in the case of the transformant bearing the multicopy vector (679[pYS32N]). Even though we saw a sixfold increase in D-xylulokinase activity in the strain bearing the multicopy plasmid, this strain showed a slight decrease (ca. 15%) in the volumetric uptake rate of D-xylulose and a moderate decrease (ca. 30%) in volumetric ethanol production compared to the strain bearing a single-copy vector. The specific xylulose consumption rates of the two strains were comparable (0.61 and 0.62 g of xylulose per g of cells per h, respectively).

DISCUSSION

The *P. stipitis* gene for xylulokinase (*XYL3*) differs significantly from its nearest homologue, and its heterologous expression enhances xylulose fermentation by *S. cerevisiae*. This is the only xylulokinase characterized from a eukaryote that is capable of converting xylose to ethanol. The divergent structure of *XYL3* is somewhat surprising because the genes for *CYC1*, *URA3*, *LEU2*, *ADH1*, *ADH2*, *PDC1*, and *PDC2* cloned previously from *P. stipitis* show between 81 and 65% identity to their *S. cerevisiae* homologues at the protein level (5, 27–29, 40). Genes for carbohydrate metabolism, such as the *P. stipitis* sugar transporters *XYL1* and *XYL2* (2, 25, 50), show between 60 and 53% identity to their nearest known *S. cerevisiae* ho-

TABLE 2. Growth and fermentation characteristics between host and recombinant strains during xylulose fermentation

Strain	Specific growth rate (h ⁻¹)	Specific xylulose consumption rate (g of xylulose [g of cells h ⁻¹])	Max ethanol concn (g of ethanol/liter)
<i>S. cerevisiae</i> 679C	0.026	0.198	0
<i>S. cerevisiae</i> 679[pYS31N]	0.037	0.611	3.97
<i>S. cerevisiae</i> 679[pYS32N]	0.039	0.622	2.83

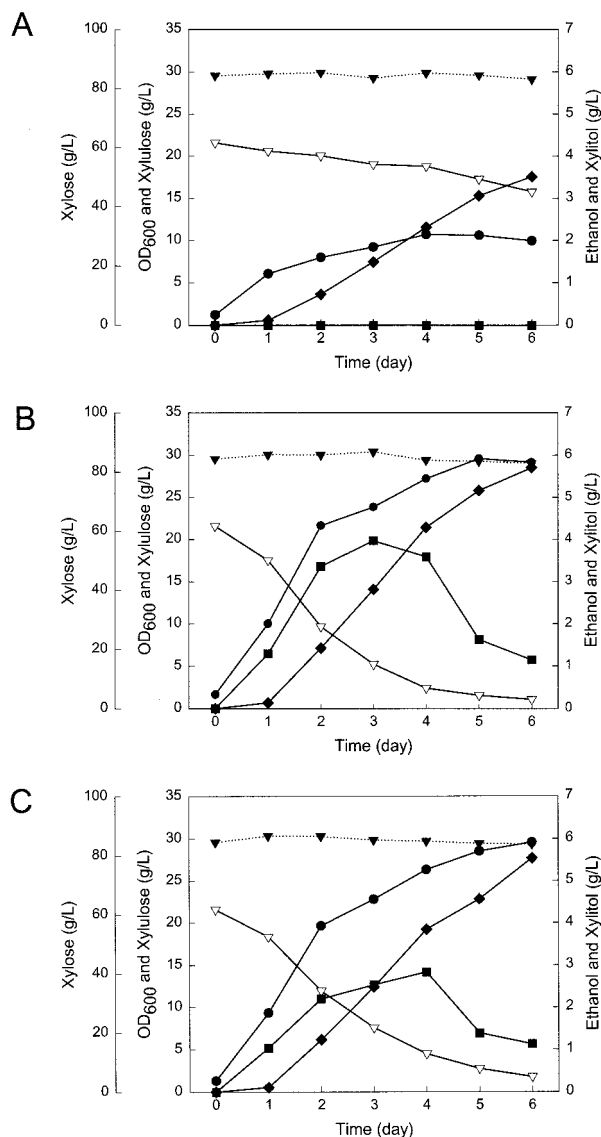


FIG. 6. Fermentation of xylose-xylulose mixture by (A) parental strain (*S. cerevisiae* 679) transformed with the multicopy control vector pRS424 and recombinant *S. cerevisiae* carrying the *XYL3* gene in the single-copy vector pYS31N (B) or the multicopy vector pYS32N (C). Cells were cultured in YSC medium with xylose-xylulose, with an oxygen transfer rate of 5.3 mM O₂ h⁻¹. Symbols: ▼, xylose; ▽, xylulose; ◆, xylitol; ●, cell density; ■, ethanol. Results in A, B, and C are each from a single representative trial.

mologues. Of the 16 *P. stipitis* enzymes with known homologues in *S. cerevisiae*, the *XYL3* product shows the least similarity to its corresponding *Saccharomyces* protein. Some *P. stipitis* genes, such as those for D-arabinitol dehydrogenase (16) and *STO1* (39), have no known *S. cerevisiae* homologues.

The amino acid sequence deduced from the open reading frame of *XYL3* had all five sequence motifs, phosphate 1 (residues 17 to 22), connect 1 (residues 304 to 310), phosphate 2 (residues 323 to 327), adenosine (residues 527 to 529), and connect 2 (residues 559 to 563), that are found in the ATP-binding pattern as defined from the multiple sequence alignment of the known members of the sugar kinases, actin, and

hsp70 class (3). Especially, the invariant GT in phosphate 2, which is present only in sugar kinases, was also found in the deduced protein sequence of *XYL3*.

The FPL-YS30.*xyl3::URA3* disruptant could grow on xylose, but only at a much lower rate than its parent, FPL-UC7 (*ura3*). On the other hand, the mutant produced no ethanol from xylose. We did not expect it to use xylose at all because disruption of *XYL3* should have eliminated xylulose phosphorylation. Its unexpected capacity for growth on xylose led us to consider two possibilities: the presence of a second isozyme for xylulokinase, and an alternative pathway for xylulose metabolism.

The presence of multiple isozymes is very common in yeasts and other higher eukaryotes. However, when we used the cloned *XYL3* fragment to probe the genomic DNA by Southern blot, only one band was observed (Fig. 3C) and the mutant did not exhibit D-xylulokinase activity. Given the divergence of *XYL3* from other known D-xylulokinases, it is possible that a second enzyme would not have been picked up in a Southern hybridization. However, because polyols, particularly arabinitol, accumulated in the culture of the *XYL3* disruptant, we suspect that xylulose phosphorylation can be bypassed via formation of arabinitol and ribulose (Fig. 7). This latter route was described by Shi et al. (41) through the characterization of a *P. stipitis xyl2* mutant that could not use D-xylose or L-arabinose but could grow on D-arabinitol, and is further evidenced by the presence of D-arabinitol dehydrogenase in *P. stipitis* (16) and by the reported production of ribitol from xylose by *P. stipitis* strains blocked with respiration inhibitors (22). Eliasson et al. (11) reported that *S. cerevisiae* engineered to overexpress *XKS1* also secretes arabinitol, but it is unclear how that observation is related to the current findings.

Even though our analytical method could not determine the stereochemistry of the arabinitol produced, the observations cited above suggest that it was D-arabinitol. Using the pro-

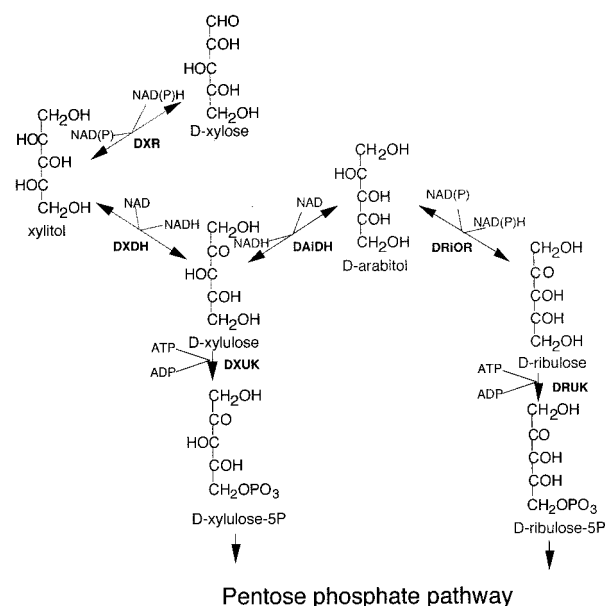


FIG. 7. Proposed bypass of D-xylulose metabolism through D-arabinitol and D-ribulose.

posed bypass, D-xylulose could be reduced to D-arabinitol by D-arabinitol dehydrogenase, and subsequently D-arabinitol could be oxidized to D-ribulose by D-ribulose reductase. Finally D-ribulose would be phosphorylated to ribulose-5-phosphate by ribulokinase, which then would enter the pentose phosphate pathway.

This route is not functional in *S. cerevisiae*, because an *XKS1* disruption mutant could not grow on xylulose and growth on xylulose was restored after complementation of *XKS1* (32). Moreover, Blast and tblastn (<http://www3.ncbi.nlm.nih.gov/BLAST/>) searches of the *S. cerevisiae* genome did not reveal any proteins with structures similar to the D-arabinitol dehydrogenase of *P. stipitis*, so it is unlikely that the bypass pathway could exist in *S. cerevisiae*. The D-xylulokinase from *S. cerevisiae* is also known to possess D-ribulokinase activity (31). The *P. stipitis* D-xylulokinase differs in that it does not possess D-ribulokinase activity and hence is more specific for D-xylulose (13).

The *XYL3* gene under its native *P. stipitis* promoter was actively expressed in *S. cerevisiae*, as shown by the observed significant increase in *in vitro* D-xylulokinase activity. This is similar to the observations for the native promoters for *P. stipitis* *XYL1* and *XYL2* expressed in *S. cerevisiae* (2, 25). We observed a copy number effect. D-Xylulokinase activity increased sixfold in the multicopy versus single-copy vector transformant, but the capacities of multi- and single-copy strains were very similar in their fermentation of D-xylulose. Ethanol production decreased with increasing copy number and increasing enzyme activity, but our results are not comparable to the deleterious effect previously observed with the overexpression of *XKS1* in *S. cerevisiae* (32). Recombinant *S. cerevisiae* strains bearing *XYL3* produced ethanol from a mixture of xylulose and xylose, whereas the parental strain could not. These results, along with the structural homologies to known bacterial D-xylulokinases and the absence of D-xylulokinase activity in the *xyl3* disruption mutant, strongly suggest that the gene product of *XYL3* is a D-xylulokinase.

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