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Characterization and Complementation of a *Pichia stipitis* Mutant Unable to Grow on D-Xylose or L-Arabinose

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Abstract

Pichia stipitis CBS 6054 will grow on D-xylose, D-arabinose, and L-arabinose. D-Xylose and L-arabinose are abundant in seed hulls of maize, and their utilization is important in processing grain residues. To elucidate the degradation pathway for L-arabinose, we obtained a mutant, FPL-MY30, that was unable to grow on D-xylose and L-arabinose but that could grow on D-arabinitol. Activity assays of oxidoreductase and pentulokinase enzymes involved in D-xylose, D-arabinose, and L-arabinose pathways indicated that FPL-MY30 is deficient in D-xylitol dehydrogenase (D-XDH), D- and L-arabinitol dehydrogenases, and D-ribitol dehydrogenase. Transforming FPL-MY30 with a gene for xylitol dehydrogenase (*PsXYL2*), which was cloned from CBS 6054 (GenBank AF127801), restored the D-XDH activity and the capacity for FPL-MY30 to grow on L-arabinose. This suggested that FPL-MY30 is critically deficient in *XYL2* and that the D-xylose and L-arabinose metabolic pathways have xylitol as a common intermediate. The capacity for FPL-MY30 to grow on D-arabinitol could proceed through D-ribulose.

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Index Entries: D-Xylose; L-arabinose; *Pichia stipitis*; fermentation; metabolism; mutant; complementation.

Introduction

D-Xylose and L-arabinose are the two principal five-carbon sugars present in hemicellulosic materials *(1)*. Current research is focused on developing a microorganism that can efficiently convert both of these sugars to fuel ethanol. Although yeasts and fungi are considered potential candidates, few are able to use L-arabinose efficiently *(1)*. In a survey of 116 yeasts selected for their abilities to catabolize L-arabinose and ferment glucose, only 4 produced detectable levels of ethanol from L-arabinose *(2)*. The maximum amount of ethanol produced was 3.4 g/L after 14 d.

The xylose-metabolizing yeast *Pichia stipitis* can use the pentose phosphate pathway (PPP) to convert xylose to significant amounts of ethanol *(3)*. *P. stipitis* can grow on L-arabinose and convert it to arabinitol *(4)*, but it has never been reported to produce significant amounts of ethanol from this sugar. It is not clear why arabinitol is not converted to ethanol even though it is used to generate cell mass. The degradation route of L-arabinose in *P. stipitis* requires clarification.

Previous studies of the metabolism of D-xylose, D-arabinose, and L-arabinose in fungi have provided insight. All three sugars are converted to their respective polyols by one or more aldose reductases. Wittveen et al. *(5)* have proposed that in *Aspergillus niger*, L-arabinose metabolism proceeds via L-arabitol and L-xylulose and connects to D-xylose metabolism with xylitol as a common intermediate (Fig. 1). In *Candida tropicalis* and *Candida albicans*, D-arabinitol dehydrogenase (D-ArDH) is the primary dehydrogenase that converts D-arabinitol to D-ribulose *(6,7)*. Following phosphorylation, D-ribulose is converted to D-ribulose-5-phosphate, and then it enters the PPP. However, L-arabinose is the primary enantiomer found in nature, and the critical steps in its degradation are not fully understood.

P. stipitis has two polyol dehydrogenases. *PsXYL2*, which codes for a medium-chain D-xylitol dehydrogenase (D-XDH), was cloned by Kötter et al. *(8)* from CBS 5774. This enzyme has its highest activity with xylitol as a substrate *(9)*. *P. stipitis* also has a short-chain dehydrogenase, which has its highest activity against D-arabinitol. However, D-ArDH activity is induced by D-arabinitol but not by L-arabinitol or D-xylose. The gene *PsArDH* was cloned by Hallborn et al. *(10)* from CBS 6054. These two structurally different dehydrogenases might perform overlapping functions in the metabolism of D-xylose in *P. stipitis (8,10)*.

To clarify the roles of these and other enzymes in the metabolism of L-arabinose and D-xylose, we used chemical mutagenesis to obtain mutants of *P. stipitis* that were deficient in the metabolism of L-arabinose and D-xylose. We then assayed the activities of the polyol dehydrogenases and other key enzymes in wild-type CBS 6054 and the mutant cells grown (or induced) on L-arabinose. Transformation of this mutant with an *XYL2* gene from

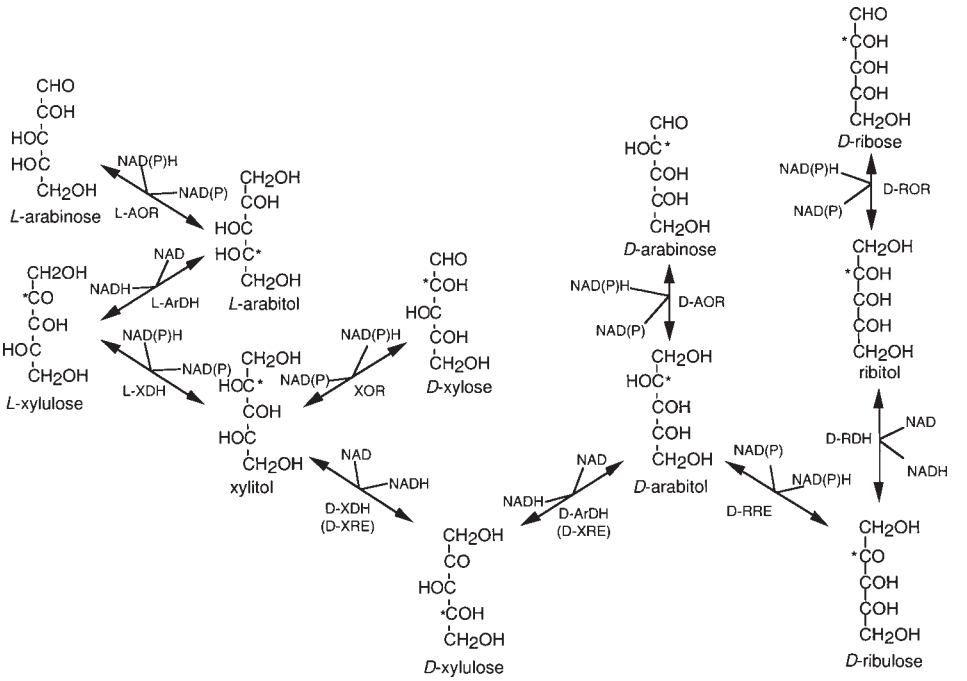

Fig. 1. The proposed connections among the L-arabinose, D-xylose, D-arabinose, and D-ribose metabolic pathways.

CBS 6054 restored growth on L-arabinose. These results implicate xylitol oxidation as a critical step that is shared in the metabolism of D-xylose and L-arabinose by *P. stipitis*.

Materials and Methods

Microbial Strains

The *P. stipitis* strains used in this study were CBS 6054 (NRRL Y-1145, ATCC 58785) and FPL-PSU1 (*ura3-2*, NRRL Y-21446). FPL-PSU1 was a Ura^- auxotroph derived from CBS 6054 by selecting for resistance to 5-fluoro-orotic acid. *Escherichia coli* DH5α (F^- *recA1 endA1 hsdR17* [rK^-, mK^+] *supE44 thi-1 gyrA relA1*) (Gibco-BRL, Gaithersburg, MD) was used for routine recombinant DNA experiments (Stratagene, La Jolla, CA). XL-1 BlueMRF' (*recA– mcrA– mcrB– mrr–*) and SOLR™ (Stratagene) strains were also used in conjunction with the *P. stipitis* λ-ZAPII recombinant phage library.

Media

Yeast nitrogen base (YNB) without amino acids (1.7 g/L) (Difco, Detroit, MI) plus $(NH_4)_2SO_4$ (5 g/L) was used for yeast cultivation and transformation. YNBX and YNBG media contained YNB plus D-xylose (20 or 40 g/L) or D-glucose (20, 40, or 80 g/L), respectively, as a carbon source. Yeast urea

peptone (YUP) medium employed YNB without amino acids (1.7 g/L) plus urea (2.27 g/L) and peptone (6.56 g/L) as nitrogen sources and L-arabinose (20 or 80 g/L) as a carbon source. Uridine was supplied at 20 mg/L for the growth of FPL-PSU1. Synthetic complete medium *(11)* was used to test for D-xylose and L-arabinose auxotrophy. Yeast extract peptone dextrose (YEPD) medium *(12)* (10 g/L of yeast extract; 20 g/L of peptone; and 20 g/L of D-glucose) or YEP-glycerol (20 g/L) was used for cultivation of mutants for enzyme assays and growth trials. *E. coli* was cultivated in Luria–Bertani (LB) medium (Difco) supplemented with 50 μg/mL of ampicillin when required.

Enzymes and Primers

Restriction enzymes and other DNA modification enzymes were obtained from New England Biolabs (Beverly, MA), Stratagene, Promega (Madison, WI), or Boehringer Mannheim (Indianapolis, IN). Reaction conditions were as recommended by the suppliers. Primers were synthesized by Ransom Hill (Romona, CA).

Nitosoguanidine Mutagenesis

P. stipitis FPL-PSU1 (*ura3-2*) cultivated on YEPD agar at 30°C for 2 d was used to inoculate 50 mL of YEPD broth in a 125-mL Erlenmeyer flask. The starting cell concentration was 1×10^6 cells/mL, and the culture was grown at 30°C with vigorous shaking (200 rpm) for 18 h. The cells were harvested at 2×10^8 cells/mL, and 2.5 mL of the cells were washed twice with potassium buffer (pH 7.0). The washed cells were then resuspended in 10 mL of the potassium buffer, and 40 μL of a nitrosoguanidine (10 mg/mL) solution were added to the tube *(13)*. Cells were incubated with nitrosoguanidine at 30°C for 1 h without shaking. The mutated cells were then plated on YEPD and cultivated at 30°C for 2 to 3 d. Colonies that appeared were then replica plated onto synthetic complete medium containing 2% D-glucose, glycerol, D-xylose, or L-arabinose and incubated for 3 to 4 d. Colonies that grew on D-glucose or glycerol but not D-xylose or D-arabinose were selected for further characterization. Cells were patched onto fresh D-glucose, glycerol, D-xylose, and L-arabinose plates and observed for reversion frequency after 2 to 5 d of incubation.

Cell Preparation for Enzymatic Assays

P. stipitis CBS 6054 was first grown in 250 mL of YEPD liquid medium at 30°C with shaking at 180 rpm for 2 d. The collected cells were washed once with sterile water and once with YUP-L-arabinose (80 g/L) medium. The cells were then resuspended in 5 mL of the same medium. One milliliter of the suspension was used to inoculate each of the six flasks containing 50 mL of fresh YUP-L-arabinose medium. The cells were grown at 25°C with shaking at 100 rpm for 64 h, harvested, and washed once with sterile 0.1 *M* MOPS buffer. They were then resuspended in a volume of 0.1 *M* MOPS

buffer (pH 7.0) equal to the weight of the decanted pellet and stored at –90°C for future use.

For comparison of enzyme activities in FPL-MY30 and FPL-PSU1, cells were first grown in YEPD (2%), and then in YEP-glycerol (2%) liquid medium for 2 d. Then they were washed and resuspended in 5 mL of fresh YUP-L-arabinose (8%), and 1 mL of this cell suspension was inoculated into six flasks containing 50 mL of YUP-L-arabinose medium (8%). The cultures were incubated at 25°C with shaking at 100 rpm for 3 h. The cells were harvested by centrifugation, and then they were washed twice and resuspended in a volume of 0.1 *M* MOPS (pH 7.0) equal to the weight of the decanted cell pellet. These cell slurries were quick-frozen and stored at –90°C.

For comparison of xylose-induced enzyme activities in FPL-MY30 (pXYL2) and FPL-MY30(pJM6), transformants were first grown in YNBG (4%) for 2 d. The transformants were then grown in YNBX (4%) overnight, and finally the cells were harvested for enzyme activity assays.

Preparation of Cell-Free Extracts

Cells in 0.5 mL of 0.1 *M* MOPS buffer (pH 7.0) were broken by vortexing with 0.5 g of 425- to 600-μ glass beads in a borosilicate glass tube. Cells were vortexed vigorously for three or four 1-min bursts, with cooling on ice for at least 1 min between bursts *(14)*. Then the tube was spun at $1.6 \times 10^4 g$ for 20 min, and the supernatant solution was transferred to a new tube on ice. All enzyme assays were performed within 4 h of cell breakage by using a Hewlett Packard 8452A spectrophotometer (Wilmington, DE) with a temperature control panel.

Pentose Reductases

The assay was conducted in a 1-mL mixture containing 50 m*M* sodium phosphate buffer (pH 6.5); 0.2 m*M* NADH or NADPH, 100 m*M* D-xylose, D- or L-arabinose, or D-ribose; and 10 to 100 mL of cell extract. The reaction was conducted at 30°C. The reaction was monitored by following the rate of NADH or NADPH oxidation at 340 nm *(5)*.

Polyol Dehydrogenases

The assay was performed in a 1-mL mixture containing 100 m*M* glycine (pH 9.6); 0.4 m*M* NAD^+ or $NADP^+$; 100 m*M* xylitol, D- or L-arabinitol, or D-ribitol; and 10–100 μL of cell extract at 30°C. The reaction was monitored by following the reduction of NAD or NADP at 340 nm *(7)*.

Pentulose Reductases

The assay was performed in a 1-mL mixture containing 50 m*M* sodium phosphate buffer (pH 6.5), 0.2 m*M* NADPH or NADH, 5 m*M* D- or L-xylulose or D-ribulose, and 10–100 μL of cell extract at 30°C. The reaction was monitored by following the rate of NADH or NADPH oxidation at 340 nm *(5)*.

Pentulose Kinases

The assay was monitored by coupling the reduction of pyruvate to lactate with lactate dehydrogenase. Pentulose kinase provides rate-limiting amounts of adenosine 5'-diphosphate to allow pyruvate kinase to convert phosphoenolpyruvate (PEP) into lactate. The reaction was conducted in a 1-mL mixture containing 250 m*M* glycylglycine (pH 7.4), 5 m*M* $MgSO_4$, 0.2 m*M* NADH, 1.5 m*M* PEP, 1 m*M* ATP, 1 U of pyruvate kinase, 3 U of lactate dehydrogenase, and 5 m*M* D-xylulose or D- or L-ribulose. The assay was performed at 30°C by monitoring the rate of NADH oxidation at a wavelength of 340 nm *(5)*.

Library Screening

A 1963-bp *XYL2* fragment from *P. stipitis* CBS 6054 was amplified in a polymerase chain reaction (PCR) *(15)*. The PCR primers were (1) 5'-CTC GAGCTCTCTAGACCACCCTAAGTC-3', and (2) 5'-CTCGAGCTCGGA TCCACT ATAGTCGAA-3' containing a *Sac*I site at each end. These primers were based on the D-XDH gene sequence of *P. stipitis* CBS 5774 *(9)*. The reaction conditions included one initial cycle at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 2 min at 50°C, and 3 min at 75°C. The reaction was terminated at 75°C for 15 min. This *XYL2* fragment labeled with digoxiginin was then used to screen a *P. stipitis* CBS 6054 λ-ZAP II library *(16)*. Plaque hybridization was conducted using a Genius1™ kit (Boehringer Mannheim) with Nytran filters (Schleicher & Schuell, Keene, NH). Hybridizations were performed in 25% formamide at 37°C. Vector pBluescript SK(–) (Stratagene, La Jolla, CA) was used to harbor the DNA from the positive clones. Posthybridization washes were done for 15 min at 37°C.

DNA Sequencing and Analysis

The nucleotide sequence of the *XYL2* gene was sequenced using a 377 ABI automated DNA sequencer (PE Biosystems, Foster City, CA). DNA sequence assembly, alignment, and analysis were conducted by using the DNAMAN (Lynnon BioSoft, Quebec, Canada) analysis software and GCG software package *(17)*. BLAST searches were performed on the National Center for Biotechnology Information server.

Complementation

A 2.1-kb *Hind*III-*Hind*III insert fragment was excised from a positive clone obtained from the library screening. The overlaps were filled in using DNA polymerase I (Klenow) to create a blunt end *(18)*. This fragment was then subcloned into the *Sma*I site of pJM6 *(19)* to create plasmid pXYL2. Plasmids pXYL2 and pJM6 were used to transform FPL-MY30, and putative transformants were picked after 4 d on YNBG (4%) plates. Complementation of the mutational deficiency was determined by assaying for

D-XDH activity and by cell growth. For enzyme assays, the transformed FPL-MY30(pXYL2) test and FPL-MY30(pJM6) control strains were cultivated on a YNBG (4%) plate for 2 d, and then the cells were collected and washed twice with sterile water. The cells were transferred to YNBX (2%) liquid medium overnight. The cells were harvested and resuspended in 0.1 *M* MOPS buffer (pH 7.0) for the D-XDH assay. For the growth experiment, the FPL-MY30(pXYL2) test and FPL-MY30(pJM6) control strains were first cultivated on YNBG (2%) plates for 3 d. The cells were then washed from the agar surface and transferred to 25 mL of YUP-L-arabinose (2%) or YUP D-arabinitol (2%) liquid media in 125-mL Erlenmeyer flasks and incubated at 30°C with shaking at 100 rpm.

DNA Isolation and Transformation

Yeast genomic DNA was isolated by the method described in Rose et al. *(12)* except Novozyme 234 (Novo Nordisk, Franklinton, NC) was used instead of Zymolyase 100,000. The volumes were also changed. The cells were resuspended in 500 μL of 1 *M* sorbitol. Then 100 μL of 0.5 *M* ethylenedinitrotetraacetic acid, 35 μL of 1 *M* dithiothreitol, and 100 μL of 20 mg/mL of Novozyme 234 in 1 *M* sorbitol were added. After incubation at 37°C for approx 15–20 min, spheroplasts were resuspended in 400 μL of buffer and 40 μL of 10% sodium dodecyl sulfate. After incubation at 65°C for 30 min, 150 μL of potassium acetate were added, the solution was centrifuged, and the DNA was precipitated with isopropanol. *E. coli* DNA isolation was carried out by a Qiagen spin column kit (Qiagen, Valencia, CA). *E. coli* transformation was performed as described in Sambrook et al. *(18)*. Yeast transformation was carried out by the lithium acetate method as described in Rose et al. *(12)* or by electroporation *(19)*. Twenty micrograms of DNA were digested with *Sac*I and separated on 1% TBE gel. Southern hybridization was performed according to Sambrook et al. *(18)* to confirm the putative transformants. DNA was transferred to a Nytran filter (Schleicher & Schuell) and probed with a 1.2-kb *PsURA3* gene from pVY3 *(18)*. Hybridization was performed in 25% formamide at 37°C.

Fermentation Studies

FPL-MY30 and FPL-PSU1 were grown on YNB plates containing either 2% D-xylose or D-glucose for 3 d. The cells were then washed with sterile water and used to inoculate 50 mL of YNB medium with 8% xylose, 8% glucose, or 4% xylose plus 4% glucose *(20)* in a 125-Erlenmeyer flask. The cultures were grown at 25°C with shaking at 100 rpm. One milliliter samples were withdrawn daily from each flask. Growth was determined by measuring light scattering at 600 nm after diluting samples to obtain apparent optical densities (ODs) between 0.05 and 0.5. ODs were converted to dry wt with the correlation of 1 OD_{600} = 0.21 g/L. Then the samples were spun for 5 min at $1.6 \times 10^3 g$. The supernatant solutions were used for high-performance liquid chromatography (HPLC) or gas chromatography

analysis as described in Shi et al. *(20)* to determine the sugar consumption and ethanol production rates.

Results and Discussion

Enzymatic Activities of CBS 6054 Cells Grown on L-Arabinose

To gain primary insight into the L-arabinose metabolism in *P. stipitis*, we assayed cell homogenates of cells grown on L-arabinose for activities of the key enzymes involved in the metabolic pathways of D-xylose and L-arabinose. We used both NAD(H) and NADP(H) in assaying activities with each substrate. When *P. stipitis* CBS 6054 was grown on L-arabinose, the major enzymes encoding for the xylose metabolism pathway, xylose reductase (XOR), D-XDH, and D-xylulose kinase (D-XUK) were all detected. In examining pentose reductase, we detected significant activity with L-arabinose reductase (L-AOR) but much less with D-arabinose reductase (D-AOR) as a substrate. Conversely, in examining polyol dehydrogenases, significantly more D-ArDH was present than L-arabinitol dehydrogenase (L-ArDH). Among pentulose oxidoreductase activities, D-xylulose reductase (D-XRE) was much higher than L-xylulose reductase (L-XRE). D-Ribitol dehydrogenase (D-RDH) and D-ribulose reductase (D-RRE) were both high even though D-ribose reductase was low when cells were grown on L-arabinose (Table 1).

Cofactor specificity followed a consistent pattern. NADPH-dependent XOR activity was 1.7-fold higher than the NADH-dependent activity; the NADH/NADPH ratio was 0.6. This was very close to the NADH/NADPH ratio (0.7) previously observed with xylose-grown CBS 6054 cells *(21,22)*. D-ribose reductase (D-ROR) and L-AOR activities also showed dual cofactor specificities with NADH/NADPH ratios of about 0.5–0.7 as well. All of the polyol dehydrogenase activities were predominantly NAD dependent. D-RDH activity was considerably higher than other polyol dehydrogenases.

Converse to the pentose reductase activities, the pentulose reductases showed higher activities with NADH than with NADPH as the cofactor. Interestingly, L-XDH was the lowest of the pentulose reductase activities observed even though the cells were grown on L-arabinose. The high D-XRE activity observed with NADH could be attributed in part to the fact that this reaction forms both xylitol and D-arabitol.

Kinase activities were consistent with D-XUK as the primary phosphorylation stem. However, the D-ribulokinase (D-RUK) activity was ninefold higher than the L-RUK activity, even though the cells were grown on L-arabinose.

The enzymes encoding for L-arabinose metabolism were detected in CBS 6054 grown on L-arabinose. D-AOR activity was 25- to 30-fold lower when compared to L-AOR activity (Table 1). This indicated that the presence of L-arabinose did not induce D-AOR. Interestingly, D-ArDH activity

Table 1
Enzymatic Activities of the Key Pentose-Metabolizing Enzymes in *P. stipitis* CBS 6054 Grown on L-Arabinose

Assay	Enzymatic activity (IU/mg protein) Cofactor	
Pentose reductases	NADH	NADPH
D-Xylose reductase (D-XOR)	0.62 ± 0.001	1.07 ± 0.03
L-Arabinose reductase (L-AOR)	0.60 ± 0.01	1.23 ± 0.05
D-Arabinose reductase (D-AOR)	0.02 ± 0.01	0.05 ± 0.01
D-Ribose reductase (D-ROR)	0.16 ± 0.01	0.23 ± 0.01
Polyol dehydrogenases	NAD	NADP
D-Xylitol dehydrogenase (D-XDH)	1.48 ± 0.13	0.03 ± 0.02
L-Arabinitol dehydrogenase (L-ArDH)	0.12 ± 0.01	0.01 ± 0.01
D-Arabinitol dehydrogenase (D-ArDH)	0.69 ± 0.01	0.01 ± 0.01
D-ribitol dehydrogenase (D-RDH)	2.40 ± 0.11	0.02 ± 0.01
Pentulose reductases	NADH	NADPH
D-Xylulose reductase (D-XRE)	9.23 ± 1.30	0.76 ± 0.20
L-Xylulose reductase (L-XRE)	0.11 ± 0.01	0.04 ± 0.01
D-Ribulose reductase (D-RRE)	2.36 ± 0.11	0.45 ± 0.11
Pentulose kinases		
D-Xylulose kinase (D-XUK)	14.7 ± 0.65	
D-Ribulose kinase (D-RUK)	7.18 ± 0.41	
L-Ribulose kinase (L-RUK)	0.80 ± 0.27	

was sixfold higher than L-ArDH activity, even though cells were grown on L-arabinose. L-ArDH and L-XDH were present in low titers. These results indicated that both D-xylose and L-arabinose pathways might share some common enzymatic steps but that the two putative intermediate steps for L-arabinose metabolism are not highly active.

We used HPLC to perform sugar analysis on the final products from the pentulose reductase assays. Taylor et al. *(23)* found that D-arabinitol is produced when CBS 6054 is grown on D-xylulose. In our study, when L- or D-xylulose was used as the substrate in enzymatic assays, arabinitol and xylitol were the primary products. Hallborn et al. *(10)* showed that when D-arabinitol is used as a substrate, D-ribulose is the final product. In our case, when D- or L-ribulose was used as the substrate, arabinitol was the primary final product. The analytical methods we used could not resolve D- and L-arabinitol. However, our HPLC results were in agreement with the previous observations. These data support our proposed connections among the L-arabinose, D-xylose, D-arabinose, and D-ribose metabolic pathways (Fig. 1).

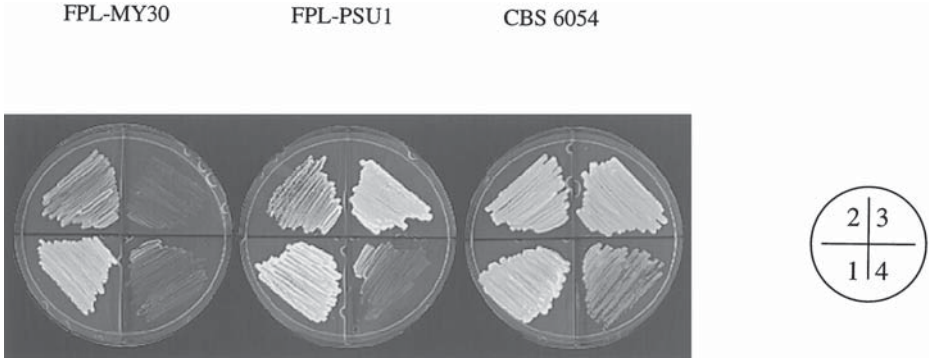

Fig. 2. Growth of FPL-MY30 and its parental strains on various carbon sources. The plates are divided into four sectors by the manufacturer. 1, YNB medium containing 2% glucose; 2, YNB medium containing 2% glycerol; 3, YNB medium containing 2% xylose; 4, YNB medium containing 2% L-arabinose. Uridine (20 mg/L) was supplied in all plates.

Transformation of FPL-MY30 with PsXYL2

To understand the roles of D-XDH and D-ArDH, we attempted to generate mutants that were blocked in either L-arabinose or D-xylose metabolism. Xylose-negative mutants with 3-fold lower XOR activity or 20-fold lower D-XDH activity were isolated previously by other researchers *(24)*. These mutants are deficient at either the *XYL1* or *XYL2* locus, respectively. Because the strains lacked a genetic transformation system, none of those mutants were complemented. In our study, we started with a parental strain, FPL-PSU1. This strain is deficient in *URA3* and can be transformed. By replica plating the mutagenized cell suspension onto various carbon sources, we identified two mutant strains that could not grow on D-xylose or L-arabinose. One of the strains, FPL-MY30, proved to be quite stable and was used for the subsequent experiments. FPL-MY30 could grow on D-glucose or glycerol, but could not grow on D-xylose or L-arabinose (Fig. 2).

Enzymatic Activities in FPL-MY30

We performed enzymatic activity assays in FPL-MY30 to determine the nature of the defect. Previous reports suggested that D-glucose represses the D-xylose metabolic pathway in *P. stipitis (25)*, so glycerol was used as the carbon source when preparing FPL-MY30 cells for the assays. Former studies suggested that even though L-arabinose pathway mutants could not grow on D-xylose or L-arabinose, enzymes for L-arabinose metabolism could be induced when the pregrown cells were transferred to L-arabinose medium *(26)*. In the present experiment, FPL-MY30 cells grown in glycerol medium for 2 d were cultivated in L-arabinose medium for 3 h prior to the assays. The parental strain, FPL-PSU1, was used as the control in this study. The XOR activity from FPL-MY30 was detected with an NADH/NADPH ratio of 1 (Table 2). The NADH-coupled activity of FPL-MY30 was the same

Table 2
Enzymatic Activities of the Key Pentose-Metabolizing Enzymes in FPL-MY30 and FPL-PSU1 Grown on Glycerol and Induced on L-Arabinose

	Enzyme activity (IU/mg protein)			
	FPL-PSU1		FPL-MY30	
Assay	NADH	NADPH	NADH	NADPH
D-XOR	0.07 ± 0.01	0.17 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
L-AOR	0.09 ± 0.01	0.24 ± 0.01	0.10 ± 0.01	0.09 ± 0.01
D-AOR	0.01 ± 0.01	<0.01	ND[a]	0.01 ± 0.01
D-ROR	0.02 ± 0.01	0.04 ± 0.01	0.02 ± 0.00	0.02 ± 0.01
D-XDH	0.47 ± 0.03	0.01 ± 0.01	ND	ND
L-ArDH	<0.01	0.01 ± 0.01	ND	ND
D-ArDH	0.17 ± 0.01	<0.01	ND	ND
D-RDH	0.46 ± 0.02	0.01 ± 0.01	ND	ND
D-XRE	1.15 ± 0.16	0.11 ± 0.04	<0.01	ND
L-XRE	0.03 ± 0.01	0.01 ± 0.01	<0.01	<0.01
D-RRE	0.46 ± 0.002	0.04 ± 0.01	<0.01	0.04 ± 0.01
D-XUK	2.76 ± 0.07		0.81 ± 0.21	
D-RUK	1.47 ± 0.15		0.72 ± 0.03	
L-RUK	1.30 ± 0.30		0.66 ± 0.06	

[a]Not detected.

as observed in the parent, FPL-PSU1. Surprisingly, the D-XDH or L- or D-ArDH and D-RDH activities were not detected in FPL-MY30. Deficiencies in D-XDH and D-RDH were most conspicuous. Control activities of L-ArDH and D-ArDH activities in the parent FPL-PSU1 strain were present but low. D-XRE and D-RRE activities in PSU1 were also greatly reduced as compared to CBS 6054. These differences could be attributed to the different induction conditions used for cultivating the CBS 6054 and the mutant yeast strains. In addition to changes in polyol dehydrogenase levels, FPL-MY30 exhibited a 3.4-fold lower D-XUK activity, and a 2-fold lower D-RUK or L-RUK activity when compared to FPL-PSU1. These data suggested that FPL-MY30 is deficient in the D-xylose and L-arabinose metabolic pathways at D-XDH, D-ArDH, L-ArDH, and D-RDH.

Complementation of FPL-MY30 with PsXYL2

To determine whether FPL-MY30 is deficient in *XYL2*, we attempted to complement FPL-MY30 with *PsXYL2*, which codes for XDH. We first cloned the *XYL2* gene from CBS 6054 by using the CBS 5774 *XYL2* gene as a probe *(9)*. One positive clone was identified, and DNA sequencing of this clone revealed a 1089-bp open reading frame that codes for a protein of 363 amino acids. This gene differs from the *XYL2* gene previously cloned from CBS 5775 in only six nucleotides. Our clone from CBS 6054 also has an

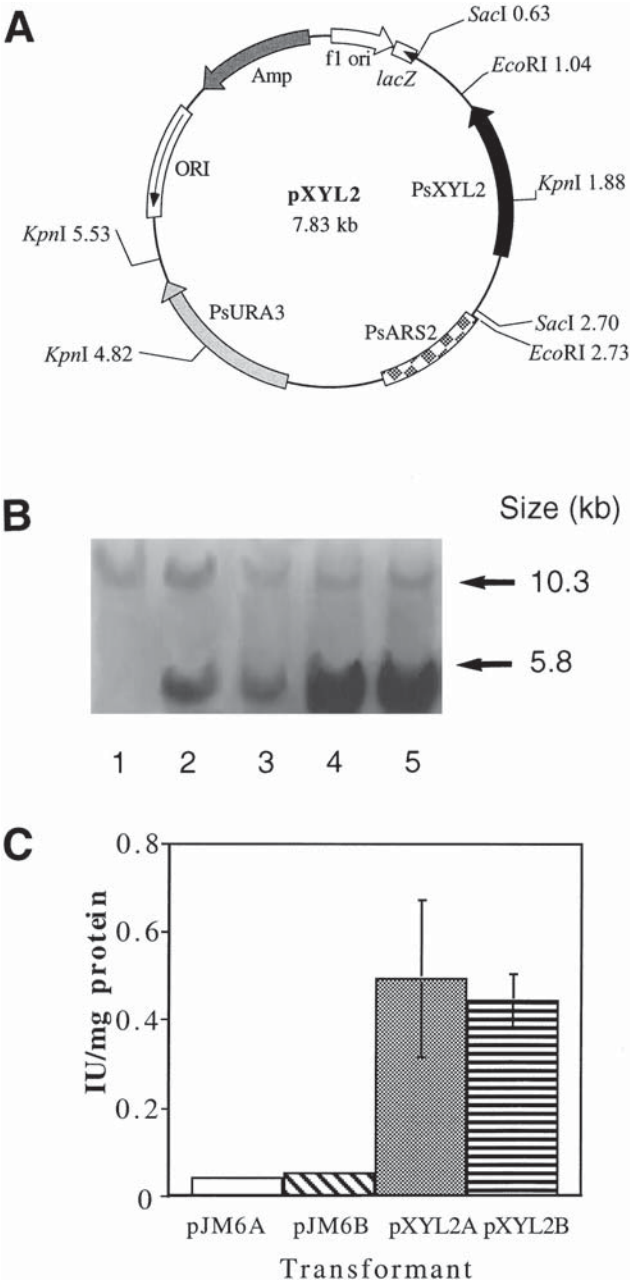

Fig. 3. **(A)** Plasmid map for pXYL2. **(B)** Genomic Southern blot for confirming the true transformants of FPL-MY30(pXYL2) and control transformant FPL-MY30 (pJM6). Lane 1, untransformed FPL-MY30; lanes 2 and 3, FPL-MY30(pXYL2); lanes 4 and 5, FPL-MY30(pJM6). All genomic DNA was digested by restriction enzyme *Sac*I. The blot was probed with a 1.2-kb *PsURA3* gene from pVY3. Arrows indicate the endogenous copy (10.3 kb) and the episomal copy of *PsURA3* (5.8 kb). **(C)** XDH activity measured from the FPL-MY30(pXYL2) and FPL-MY30(pJM6) strains. Activity was determined from three replications and represented as international units per milligram of protein.

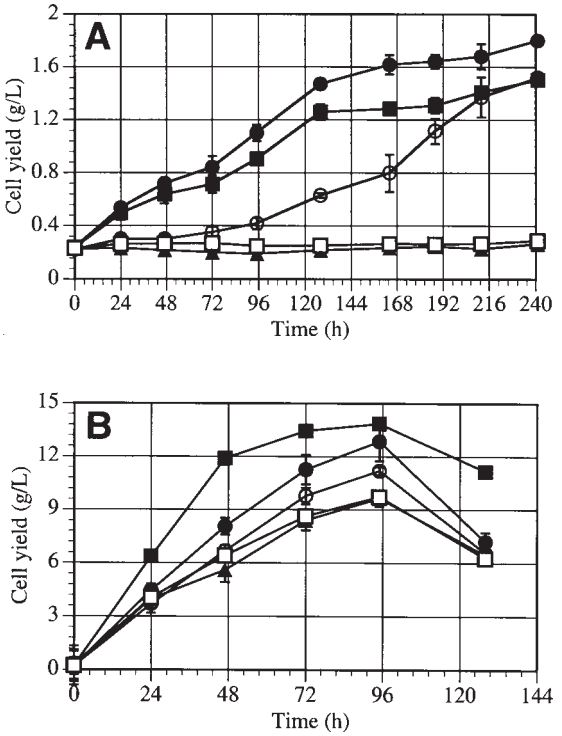

Fig. 4. **(A)** Growth curves of five different strains of *P. stipitis* on YNB-L-arabinose (2%). **(B)** Growth curves of five different strains of *P. stipitis* on YNB-D-arabinitol (2%). Growth rate was monitored as OD at OD_{600}. One OD_{600} was converted to 0.21 g cells/L (dry wt). ■, CBS 6054; ●, FPL-PSU1; □, FPL-MY30; ○, FPL-MY30 (pXYL2); ▲, FPL-MY30(pJM6).

additional 318-bp 5' untranslated sequence. A 2.1-kb insert fragment containing the full-length CBS 6054 *XYL2* gene was excised and subcloned into pJM6 *(18)* to create pXYL2 (Fig. 3A). Putative transformants of FPL-MY30(pXYL2) were confirmed by Southern blot using *PsURA3* as a probe. The transformants of FPL-MY30(pXYL2) showed an episomal band (5.8 kb) and the endogenous band (10.3 kb) of the *URA3* gene when the DNA was digested with *Sac*I (Fig. 3B). The intensity of the episomal band of *URA3* could be different owing to variance in copy number *(27)*. Transformants of FPL-MY30(pJM6) also showed the episomal and endogenous bands of *URA3*. However, the untransformed control only showed the endogenous band (10.3 kb). One of the FPL-MY30(pXYL2) strains was assayed for D-XDH activity. FPL-MY30(pXYL2) displayed D-XDH activity at 0.48 IU/mg of protein (Fig. 3C), which was 10-fold higher than the control FPL-MY30(pJM6).

We then tested whether the complementation would also restore the growth of FPL-MY30(pXYL2) on L-arabinose and D-arabinitol. CBS 6054 and the parent, FPL-PSU1, could grow on L-arabinose whereas FPL-MY30 did not grow on L-arabinose during the entire trial. By comparison, the transformed strain, FPL-MY30(pXYL2), was able to grow three generations on L-arabinose within 212 h (Fig. 4A). The control FPL-MY30(pJM6) strain

Table 3
Fermentation Results from FPL-MY30 and FPL-PSU1 on Glucose and Xylose[a]

Fermentation parameter	FPL-PSU1			FPL-MY30		
	8% G	8% X	4% G + 4% X	8% G	8% X	4% G + 4% X
Biomass yield ($Y_{X/S}$)[b]	0.26	0.21	0.22	0.17	0	0.21
Ethanol yield ($Y_{P/S}$)[c]	0.40	0.41	0.36	0.42	0	0.35

[a]Because FPL-MY30 could not produce ethanol from xylose, the numbers represent either cell yield or ethanol yield from only 4% glucose. G, glucose; X, xylose.
[b]$Y_{X/S}$ (g [dry wt] · g xylose^{-1}).
[c]$Y_{P/S}$ (g ethanol · g xylose^{-1}).

did not show any growth in the experiment. The lag in growth observed with FPL-MY30(pXYL2) could be owing to the slow conversion of L-arabinose to arabinitol. All strains used L-arabinose slowly.

Because the D-ArDH activity was greatly induced by L-arabinose, we decided to test the growth rates of FPL-MY30(pXYL1) and the control strains on D-arabinitol as a carbon source. CBS 6054 converted almost all the D-arabinitol into cell mass within 72 h (Fig. 4B). FPL-PSU1 metabolized D-arabinitol slower but also used it all within 95 h. The mutant strain, FPL-MY30, utilized D-arabinitol slower than its parent, FPL-PSU1, but in contrast to its performance on L-arabinose, it was able to grow. The cell yield of FPL-MY30 was 1.3-fold lower than that of FPL-PSU1. This observation indicated that D-ArDH could be at a lower titer in FPL-MY30. Interestingly, the complemented strain, FPL-MY30(pXYL2), showed 1.2-fold higher cell yield than FPL-MY30. This result indicated that complementing FPL-MY30 with *PsXYL2* was able to compensate for D-ArDH activity. The control strain, FPL-MY30(pJM6), which did not carry the *XYL2* expression cassette, showed the same cell yield as observed from FPL-MY30. These results not only confirmed the mutant phenotype of FPL-MY30, but also showed an important difference between the roles of D-XDH and D-ArDH.

FPL-MY30 Fermentation Results

Previously isolated D-XDH mutants appeared to accumulate xylitol after switching from glucose medium to xylose medium within 24 h *(24)*. A fermentation trial (Table 3) was carried out to study whether FPL-MY30 could produce xylitol in the presence of D-glucose. FPL-MY30 and its parental strain, FPL-PSU1, and CBS 6054 were tested in three different media. FPL-MY30 did not produce any ethanol from D-xylose when it was used as a sole carbon source during the trial. Surprisingly, the ethanol yield from FPL-MY30 on D-glucose was higher than the parent, FPL-PSU1. When 4% xylose and 4% glucose were both present, this mutant was able to produce 11 g/L of xylitol in the presence of glucose but could not continue once the glucose was completely consumed. In addition to the xylitol, FPL-MY30 produced 16 g/L of ethanol from 4% glucose. These results, along with the

enzyme assays and complementation studies, also indicated that FPL-MY30 is a mutant deficient in D-XDH. The production of significant amounts of xylitol indicated that a redox imbalance might have occurred in the cells *(28)*. Future expression of the *PsArDH* gene to complement FPL-MY30 could confirm the observations in our study.

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References

1. McMillan, J. D. and Boynton, B. L. (1994), *Appl. Biochem. Biotechnol.* **45/46,** 569– 584.
2. Dien, B. S., Kurstzman, C. P., Saha, B. C., and Bothast, R. J. (1996), *Appl. Biochem. Biotechnol.* **57/58,** 233–242.
3. Jeffries, T. W. (1985), *Trends Biotechnol.* **3,** 203–212.
4. Du Preez, J. C., Bosch, M., and Prior, B. A. (1986), *Enzyme Microbiol. Technol.* **8,** 360–364.
5. Witteveen, C. F. B., Busink, R., van de Vondervoort, P., Dijkema, C., Swart, K., and Visser, J. (1989), *J. Gen. Microbiol.* **135,** 2163–2171.
6. Quong, M. W., Miyada, C. G., Switchenko, A. C., and Goodman, T. C. (1993), *Biochem. Biophys. Res. Commun.* **196,** 1323–1329.
7. Wong, B., Murray, J. S., Castellanos, M., and Croen, K. D. (1993), *J. Bacteriol.* **175,** 6314–6320.
8. Kötter, P., Amore, R., Hollenberg, C. P., and Ciriacy, M. (1990), *Curr. Genetics* **18,** 493–500.
9. Persson, B., Hallborn, J., Walfridsson, M., Hahn-Hägerdal, B., Keranen, S., Penttiam, M., and Jornvall, H. (1994), *FEBS Lett.* **324,** 9–14.
10. Hallborn J., Walfridsson, M., Penttila, M., Keranen, S., and Hahn-Hägerdal, B. (1995), *Yeast* **11,** 8390–847.
11. Kaiser, C., Michaelis, S., and Mitchell, A. (1994), *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, New York.
12. Rose, M. D., Winston, F., and Hieter, P. (1990), *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, New York.
13. Lawrence, L. (1991), *Methods Enzymol.* **194,** 273–281.
14. Ciriacy, M. (1975), *Mutat. Res.* **29,** 315–326.
15. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988), *Science* **239,** 487–491.
16. Lu, P., Davis, B. P., and Jeffries, T. W. (1998), *Appl. Environ. Microbiol.* **64,** 94–97.
17. Devereux, J., Haeberli, P., and Smithies, O. (1984), *Nucleic Acids Res.* **12,** 387–395.
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.
19. Yang, V. W., Marks, J. A., Davis, B. P., and Jeffries, T. W. (1994), *Appl. Environ. Microbiol.* **60,** 4245–4254.
20. Shi, N. Q., Davis, B., Sherman, F., Cruz, J., and Jeffries, T. W. (1999). *Yeast* **15,** 1021–1030.

21. Dahn K. M., Davis, B. P., Pittman, P. E., Kenealy, W. R., and Jeffries, T. W. (1996), *Appl. Biochem. Biotechnol.* **57,** 267–276.
22. Metzger, M. H. and Hollenberg, C. P. (1995), *Eur. J. Biochem.* **228,** 50–54.
23. Taylor, K. B., Beck, M. J., Huang, D. H., and Sakai, T. T. (1990), *J. Indus. Microbiol.* **6,** 29–41.
24. Hagedorn, J. and Ciriacy, M. (1989), *Curr. Genetics* **16,** 27–33.
25. Amore, R., Wilhelm, M., and Hollenberg, C. P. (1989), *Appl. Microbiol. Biotechnol.* **39,** 351–357.
26. De Vries, R. P., Plipphi, M. J. A., Witteveen, C. F. B., and Visser, J. (1994), *FEMS Microbiol. Lett.* **123,** 83–90.
27. Shi, N. Q. and Jeffries, T. W. (1998), *Appl. Microbiol. Biotechnol.* **50,** 339–345.
28. Jeppsson, H., Alexander, N. J., and Hahn-Hägerdal, B. (1995), *Appl. Environ. Microbiol.* **61,** 2596–2600.