

## *Pichia stipitis* Genes for Alcohol Dehydrogenase with Fermentative and Respiratory Functions

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**Two genes coding for isozymes of alcohol dehydrogenase (ADH); designated *PsADH1* and *PsADH2*, have been identified and isolated from *Pichia stipitis* CBS 6054 genomic DNA by Southern hybridization to *Saccharomyces cerevisiae* ADH genes, and their physiological roles have been characterized through disruption. The amino acid sequences of the PsADH1 and PsADH2 isozymes are 80.5% identical to one another and are 71.9 and 74.7% identical to the *S. cerevisiae* ADH1 protein. They also show a high level identity with the group I ADH proteins from *Kluyveromyces lactis*. The PsADH isozymes are presumably localized in the cytoplasm, as they do not possess the amino-terminal extension of mitochondrion-targeted ADHs. Gene disruption studies suggest that PsADH1 plays a major role in xylose fermentation because *PsADH1* disruption results in a lower growth rate and profoundly greater accumulation of xylitol. Disruption of *PsADH2* does not significantly affect ethanol production or aerobic growth on ethanol as long as *PsADH1* is present. The PsADH1 and PsADH2 isozymes appear to be equivalent in the ability to convert ethanol to acetaldehyde, and either is sufficient to allow cell growth on ethanol. However, disruption of both genes blocks growth on ethanol. *P. stipitis* strains disrupted in either *PsADH1* or *PsADH2* still accumulate ethanol, although in different amounts, when grown on xylose under oxygen-limited conditions. The *PsADH* double disruptant, which is unable to grow on ethanol, still produces ethanol from xylose at about 13% of the rate seen in the parental strain. Thus, deletion of both *PsADH1* and *PsADH2* blocks ethanol respiration but not production, implying a separate path for fermentation.**

Reduction of acetaldehyde to ethanol is the last step in ethanol production. Conversely, ethanol oxidation is the first reaction of ethanol respiration. The enzymes catalyzing these reactions and their structural genes have been well studied in *Saccharomyces cerevisiae*. Alcohol dehydrogenase (EC 1.1.1.1), ADH1, the classical fermentative isozyme, is responsible for the last step in the yeast glycolytic pathway, the reduction of acetaldehyde to ethanol (4, 8, 11). ADH2, the oxidative isozyme, is highly repressed by fermentative conditions (12) and is derepressed in the absence of a fermentable sugar such as glucose. The function of ADH2 in the cell is to oxidize ethanol, formed during fermentation, to acetaldehyde, which can then be metabolized via the tricarboxylic acid cycle in the mitochondria and also serve as an intermediate in gluconeogenesis (30). *S. cerevisiae* has two other ADH genes: *ADH3*, coding for a mitochondrion-targeted enzyme presumably involved in ethanol oxidation (48), and *ADH4*, which displays no significant similarity to any other characterized yeast ADH gene (46). Four such ADHs have been characterized in the galactose-fermenting yeast *Kluyveromyces lactis* (23, 32, 33, 38). These genes, named *KLADH1* through *KLADH4*, encode two cytoplasmic (*KIADH1* and *KIADH2*) and two mitochondrial (*KIADH3* and *KIADH4*) activities. *KLADH1* and *KLADH2* are preferentially expressed in the presence of glucose (33, 38). By contrast, *KLADH4* is induced at the transcriptional level in the presence of ethanol (23, 38). Three of the ADH isozymes from *S. cerevisiae* and the four isozymes of *K. lactis* all belong to the group I zinc-dependent enzymes because of their sequence identity and functional similarities (27). The ADH enzymes of xylose-fermenting yeasts have not been examined previously.

The xylose-fermenting yeast *Pichia stipitis* is among the few organisms that use both xylose and glucose and exhibit a regulatory transition between respiratory and fermentative processes (19). The fundamental mechanisms by which fermentation is regulated differ profoundly in the glucose-fermenting yeast *S. cerevisiae* and the xylose-fermenting yeast *P. stipitis* (16, 19, 45). In *S. cerevisiae*, the availability of oxygen is irrelevant to fermentative metabolism. Glucose induces high levels of glycolytic enzymes and represses respiration, leading to ethanol production (4). In the Crabtree-negative yeast *P. stipitis*, oxygen limitation, rather than the presence of either glucose or xylose, induces fermentation (25, 45).

*P. stipitis* is capable of producing ethanol from xylose under anaerobic conditions because it has a single xylose reductase with dual cofactor (NADPH and NADH) specificity (1). In most yeasts and fungi, xylose reductase uses only NADPH. Xylose metabolism in yeasts proceeds via xylose reductase (XR), which catalyzes the reduction of xylose with NAD(P)H to form xylitol, and xylitol dehydrogenase (XDH), which catalyzes the oxidation of xylitol by NAD to form xylulose. Thus, a cofactor imbalance can arise under anaerobic conditions if XR uses only NADPH, XDH uses only NAD, and no mechanism exists to reduce NADP with NADH. In *P. stipitis*, XR can accept either NADPH or NADH, so a cofactor imbalance does not block xylose uptake under anaerobic conditions (7).

*P. stipitis* requires small amounts of oxygen for maximal conversion of xylose to ethanol (41). Under strictly anaerobic conditions, little ethanol is formed (19). The explanation for this oxygen effect could reside in the higher activity of its XR with NADPH. Its XDH is specific for NAD, so partial accumulation of NADP and NADH probably still occurs, despite NADH-linked XR activity in *P. stipitis* (20).

Two key fermentative enzyme activities, pyruvate decarboxylase (PDC) and ADH, are induced as *P. stipitis* becomes oxygen limited and ethanol production increases (25). How-

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TABLE 1. *P. stipitis* strains used in this study

Strain	Genotype	Origin	Reference
CBS 6054 (= NRRL Y-11545 = ATCC 58785)	Wild type	CBS <sup>a</sup>	20
FPL-PSU-1	<i>Psura3-2/Psura3-2</i>	CBS 6054	47
FPL-PLU20	<i>Psura3-3/Psura3-3 Psleu2Δ-1/Psleu2Δ-1</i>	FPL-DX26 (= NRRL Y-21304)	22
FPL-PSU218	<i>Psura3-2/Psura3-2 Psadh1::PsURA3/Psadh1::PsURA3</i>	FPL-PSU1 (= NRRL Y-21446)	This study
FPL-PLU123	<i>Psura3-3/Psura3-3 Psleu2Δ-1/Psleu2Δ-1 Psadh2::PsLEU2/Psadh2::PsLEU2</i>	FPL-PLU20	This study
FPL-PLU1209	<i>Psura3-3/Psura3-3 Psleu2Δ-1/Psleu2Δ-1 Psadh1::PsURA3/Psadh1::PsURA3 Psadh2::PsLEU2/Psadh2::PsLEU2</i>	FPL-PLU123	This study

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ever, little is known about this process in *P. stipitis*, at the molecular genetic and physiological levels. A better understanding of metabolic regulation of the genes involved in xylose fermentative metabolism is essential to further advance this field. A recent paper from our laboratory described two *P. stipitis* genes for PDC (21). The next step toward understanding the roles of fermentation and respiration in xylose metabolism is to clone and disrupt *P. stipitis* ADH genes.

The objective of the present study was to isolate and characterize the ADH genes necessary for ethanol production in *P. stipitis*. The research identified two distinct genes for ADH in *P. stipitis*. We determined the physiological roles for each and showed that one (*PsADH1*) is critically important for ethanol production.

#### MATERIALS AND METHODS

**Microbial strains.** The *P. stipitis* strains used in this research are summarized in Table 1. *P. stipitis* CBS 6054 was the source of all derived strains and of all sequenced DNA. *Escherichia coli* DH5α (Gibco BRL, Gaithersburg, Md.) ( $F^-$  *recA1 endA1 hsdR17* [ $r_k^-$   $m_k^+$ ] *supE44 thi-1 gyrA relA1*) was used for all recombinant DNA experiments that required a bacterial host.

**Media and culture conditions.** Yeasts were grown in yeast-peptone-dextrose medium consisting of yeast extract at 10 g/liter, peptone at 20 g/liter, and glucose at 20 g/liter. For cultivation of *ura3* and *leu2* auxotrophs, media were supplemented with 100 mg of uridine per liter and 100 mg of leucine per liter, respectively. *E. coli* was grown in Luria-Bertani medium (34) with 50 μg of ampicillin per ml in liquid medium or 100 μg of ampicillin per ml in solid medium. Fermentation studies were done with 1.7 g of yeast nitrogen base per liter without ammonium sulfate or amino acids (YNB; Difco, Detroit, Mich.) supplemented with Bacto Peptone at 6.56 g/liter, urea at 2.27 g/liter (2× nitrogen), and 80 g of D-xylose per liter plus leucine or uridine as needed. Cells were cultivated at 25°C in 50 ml of fermentation medium shaken in 125-ml Erlenmeyer flasks at 100 rpm.

**Plasmid constructions.** The *PsURA3* (47) and *PsLEU2* (22) selectable markers originated from *P. stipitis* CBS 6054. Plasmid Bluescript KSII<sup>+</sup> was obtained from Stratagene (La Jolla, Calif.). pJY102 (*PsADH1* disruption cassette) was built in three steps: (i) the *EcoRI* site in pUC19 was destroyed by treatment with T4 DNA polymerase (New England Biolabs, Beverly, Mass.) and ligation reactions to form pJY19; (ii) a 2.0-kbp *SacI-SacI* fragment of the *PsADH1* gene bearing a unique *EcoRI* site was inserted into the *SacI* site of pJY19 to form pJY101; and (iii) a plasmid containing the *URA3* gene of *P. stipitis*, pVY3 (47), was cut with *PstI* (New England Biolabs). The *EcoRI* linker (New England Biolabs) was inserted at the remaining *PstI* site. A 1.2-kbp *EcoRI-EcoRI* fragment carrying the *P. stipitis* *URA3* gene was inserted into a unique *EcoRI* site within the *PsADH1* gene of pJY101 to form pJY102. The resulting plasmid, pJY102, contained the 3.2-kbp *SacI-SacI Psadh1::PsURA3* fragment and was digested with *SacI* prior to transformation. pJY202 (*PsADH2* disruption cassette) was built in three steps: (i) a portion (3.5 kbp) of the 5'-flanking region was deleted from *PsADH2* to leave a unique *PstI* site in its coding region; (ii) this site was destroyed by T4 DNA polymerase, and a 2.1-kbp blunt-ended *EcoRI* fragment bearing *PsLEU2* gene was ligated into the middle of *Psadh2* to form pJY201; and (iii) a 4.5-kbp *SacI-BamHI* fragment of pJY201 bearing the *Psadh2::PsLEU2* construct was inserted into the corresponding sites of pUC18 to form pJY202. The resulting plasmid, pJY202, was digested with *SacI* and *BamHI* prior to transformation.

**Yeast transformation.** Lithium acetate transformation of *P. stipitis* PSU1 (*ura3-2/ura3-2*) and PLU20 (*ura3-3/ura3-3 leu2Δ-1/leu2Δ-1*) (22) was performed as described by Ito et al. (18). Yeast transformants were selected on YNB plus

20 g of glucose per liter without uracil or leucine when *URA3* and *LEU2* were used as selectable markers, respectively. For solid media, 20 g of agar per liter was added.

**DNA isolation.** Plasmid DNA was isolated and purified by the alkaline extraction method of Birnboim and Doly (5) or with the Qiagen Plasmid Prep kit (Qiagen Corp., Chatsworth, Calif.). Yeast genomic DNA was isolated and purified as described by Specht et al. (43) or Rose et al. (28).

**Cloning of *P. stipitis* ADH genes.** Genomic DNA from *P. stipitis* CBS 6054 was cut with *BamHI* and *SalI* and electrophoresed in 0.8% agarose. DNA corresponding to ca. 6.6 kbp was isolated from the gel slice by using Gelase (Epicentre Corp., Middleton, Wis.). The purified DNA fragment was ligated into the corresponding sites of pBluescript KSII<sup>+</sup>. The DNA library was amplified in *E. coli* DH5α, followed by electroporation using the Pulse Controller together with the Gene Pulser from Bio-Rad (Richmond, Calif.). Inserts containing ADH sequences were identified by colony hybridization to the PCR-amplified *S. cerevisiae* ADH1 coding sequences, and colonies that were positive with the probe were tested further. The second genomic library was constructed from *BamHI*- and *XbaI*-digested CBS 6054 genomic DNA as described above for the isolation of *P. stipitis* ADH1. *E. coli* transformants were screened for the presence of *S. cerevisiae* ADH2 homologous sequences by using the colony hybridization technique (34).

**Blot hybridizations.** Genomic DNA digests were electrophoresed in 0.8% agarose, blotted into Nytran filters (Schleicher & Schuell, Keene, N.H.) by standard procedures (42), and hybridized with the PCR-amplified *P. stipitis* *PsADH2* or *S. cerevisiae* ADH1 or ADH2 coding sequences labeled with digoxigenin-11-dUTP by using a Genius 1 kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Hybridizations were done at 37°C under the manufacturer's (Boehringer Mannheim) specified conditions. The colony hybridizations were performed under standard conditions (34) by using a Genius 1 kit (Boehringer Mannheim).

**DNA sequencing.** The dideoxy method of Sanger et al. (35) was used for DNA sequencing (Sequenase version 2.0 DNA sequencing kit; United States Biochemical, Cleveland, Ohio). Primers were synthesized at the University of Wisconsin—Madison Biotechnology Center and Ransom Hill Bioscience, Inc. (Ramona, Calif.). Nested deletions of pJY158, a plasmid containing the *PsADH2* gene, were sequenced from universal primers located adjacent to the multiple cloning site. The sequence was confirmed by sequencing other deletions with overlapping endpoints. The sequence of the coding region and 5'- and 3'-flanking regions is available under GenBank accession no. AF008244. The *SpeI-XbaI* fragment of pJY268, a plasmid containing *PsADH1* (see Fig. 2A), was deleted, religated, and subjected to sequence analysis by primer walking from the universal primers located adjacent to the multiple cloning site. The sequence of the coding region and the 5'- and 3'-flanking regions is available under GenBank accession no. AF008245. The Genetics Computer Group programs (13) developed at the University of Wisconsin—Madison were used to evaluate DNA and derived amino acid sequences.

**PCR screening of *PsADH* disruptants.** Amplification reactions were performed in 50 μl containing 100 ng of genomic template DNA, 200 pmol of each primer, 0.2 mM each deoxyribonucleoside triphosphate (A, T, C, and G), and 5 U of *Pfu* DNA polymerase (Stratagene) in 10 mM KCl–10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>–20 mM Tris–Cl (pH 8.8)–2 mM MgSO<sub>4</sub>–0.1% Triton X-100–100-μg/ml bovine serum albumin (the 10× reaction buffer provided with the enzyme). Times and temperatures were as follows: 5 min at 94°C, 1 min at the primer annealing temperature, and 6 min at 75°C. The reaction was completed by a 10-min incubation at 75°C. Amplification was performed in a Coy Temp Cycler II (Coy Corp., Grass Lake, Mich.). The PCR products were checked by gel electrophoresis. Primers, supplied by Genosys Biotechnologies, Inc. (The Woodlands, Tex.), were as follows (sequences are 5' to 3'): no. 50 (20-mer near the 5' end of the *LEU2* insertion at *PsADH2*), TGT-CTG-TCA-CAC-CGA-CTT-GC; no. 34 (20-mer near the 3' end of the *LEU2* insertion at *PsADH2*), TGG-CTT-CAG-AGT-CAG-CT; no. 53 (18-mer near the 5' end of the *URA3* insertion at *PsADH1*),

GCT-CTA-CAA-GGA-CAT-TCC; no. 35 (18-mer near the 3' end of the *URA3* insertion at *PsADH1*), CCT-GGT-TGG-ATT-TGA-GCG.

**Analytical methods.** Determination of ethanol, xylose, and xylitol was done as described previously (9). Cell growth was determined daily by monitoring optical density at 600 nm ( $OD_{600}$ ), and cell dry weight was estimated by diluting a suspension to  $OD_{600}$ s between 0.15 and 0.5. Under these conditions, an  $OD_{600}$  of 1.0 equals 0.24 g of cells/liter.

## RESULTS

**Identification of *PsADH* genes.** *P. stipitis* genomic DNA was digested with *Bam*HI and *Sal*I, and the fragments containing *ADH* genes were identified by Southern hybridization using *S. cerevisiae ADH1* (*ScADH1*) as a probe. *S. cerevisiae* genomic DNA was used as a control. The *P. stipitis* genome showed two bands hybridizing to *ScADH1*. As expected, the *S. cerevisiae* genome showed at least three restriction fragments hybridizing to the probe.

**Cloning of *PsADH2*.** One fragment of *P. stipitis* DNA, 6.6 kbp in length, strongly cross-hybridized to the *ScADH1* coding sequences (Fig. 1A). A second fragment, about 20 kbp in length, hybridized to a lesser extent. The *Bam*HI-*Sal*I genomic fragments corresponding to 6.6 kbp were isolated from the gel to construct a DNA library pool for screening of the *ADH* gene of *P. stipitis* with *ScADH1*. Of 300 colonies screened by colony hybridization, two positive clones contained a 6.6-kbp *Bam*HI-*Sal*I fragment which hybridized to the *ScADH1* probe following Southern blot analysis. These clones were further characterized by restriction mapping and Southern hybridizations. These DNA fragments, designated *PsADH2*, strongly hybridized to the *ScADH1* probe and showed the same restriction patterns. Staggered deletions of about 500 bp each were prepared by opening the plasmid at one end of the insert, followed by timed digestions with exonuclease III (34). The deletion fragments were hybridized to the probe to localize and sequence the *PsADH2* coding regions (Fig. 2B). The coding region of *PsADH2* was tentatively located on a fragment with 4.1 and 1.5 kbp of the 5'- and 3'-flanking sequences, respectively. It was subsequently sequenced (see below).

**Cloning of *PsADH1*.** *PsADH1* was identified by hybridizing *Bam*HI and *Xba*I digests of *P. stipitis* genomic DNA to the coding sequences of *PsADH2*, *ScADH1*, and *ScADH2* (Fig. 1B). Two fragments, 9.4 and 6.0 kbp in length, strongly cross-hybridized to *ScADH1* and *ScADH2* (Fig. 1B, lanes 6 and 9). The hybridization of *ScADH2* to the *Xba*I genomic fragments showed that a third fragment, about 4.3 kbp in length, cross-hybridized to a lesser extent to this probe (Fig. 1B, lane 8). In addition, *PsADH2* cross-hybridized more to the 9.4-kbp band than to the 6.0-kbp band (Fig. 1B, lane 3). The putative *ADH1* gene of *P. stipitis* hybridized more strongly to *ScADH1* and *ScADH2* than to *PsADH2*.

To isolate *PsADH1*, a DNA library was constructed as described previously and screened by colony hybridization with *ScADH2* as the probe, and positive colonies were tested further. The restriction map of this fragment differed from that of *PsADH2* in both the coding and flanking regions. The *PsADH1* gene was localized and oriented on the 6.0-kbp fragment by hybridization to *ScADH2* and sequence comparison with other *ADH* genes (Fig. 2A).

**DNA sequence analysis.** Analysis of the sequence of *PsADH1* revealed an open reading frame (ORF) of 1,047 nucleotides which codes for a polypeptide of 349 amino acids with a calculated molecular weight of 36,496. Potential promoter elements containing a TATA-like sequence were also found upstream from the putative ORF at position -177 to position -183 (Fig. 3A). However, very little identity can be observed between the upstream sequences of the *ADH* genes from *P.*

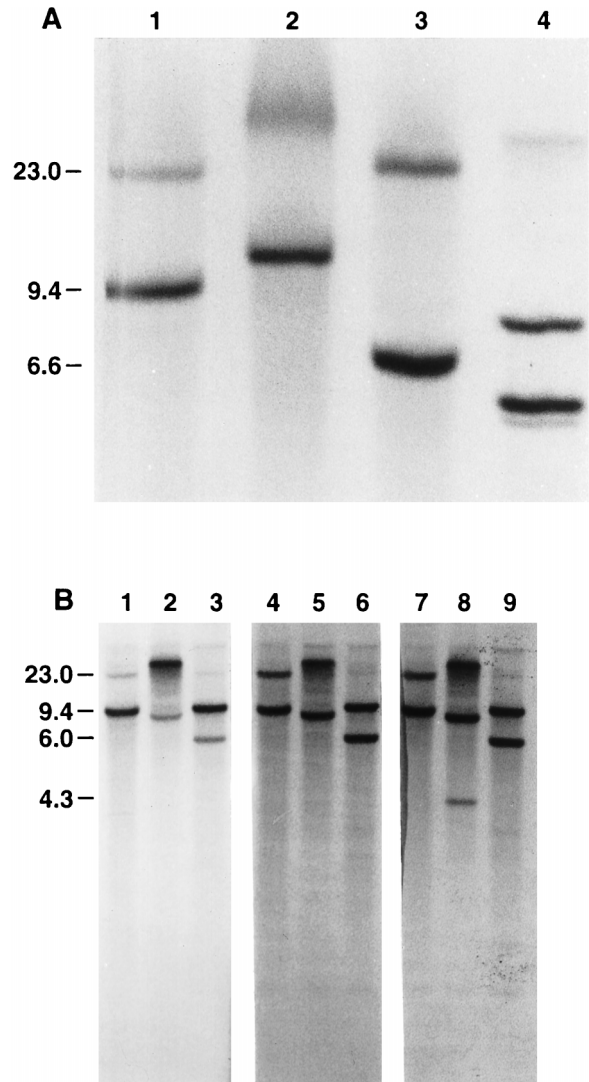


FIG. 1. Identification of *PsADH* DNA fragments by Southern blot analysis. *P. stipitis* CBS 6054 genomic DNA was digested with the restriction enzymes indicated below and hybridized with coding sequences of the *S. cerevisiae ADH1* gene (A, lanes 1 to 4; B, lanes 4 to 6), the *S. cerevisiae ADH2* gene (B, lanes 7 to 9), and the *P. stipitis ADH2* gene (B, lanes 1 to 3). *S. cerevisiae* (A, lane 4) and *P. stipitis* genomic DNAs (20  $\mu$ g of each) were digested with *Bam*HI (A, lanes 1 and 4; B, lanes 1, 4, and 7), *Sal*I (A, lane 2), *Bam*HI-*Sal*I (A, lane 3), *Xba*I (B, lanes 2, 5, and 8), or *Bam*HI-*Xba*I (B, lanes 3, 6, and 9). The *P. stipitis ADH1* (*Bam*HI-*Xba*I) and *ADH2* (*Bam*HI-*Sal*I) DNA fragments were 6.0 and 6.6 kbp long, respectively. These fragments were isolated from the gel slice. The values on the left are molecular sizes in kilobase pairs.

*stipitis* and *S. cerevisiae*. *PsADH2* contains an ORF of 1,047 nucleotides coding for a polypeptide of 349 residues and a calculated molecular weight of 36,541. The upstream sequences of *PsADH2* show a putative TATA element (consensus sequence, TATAT/AAT/A), upstream of the RNA initiation site, at position -110 to position -117 (Fig. 3B).

A comparison of the polypeptides encoded by these genes with the *ADH* polypeptides of *S. cerevisiae* and *K. lactis* showed considerable identity with the other yeast *ADH*s (Fig. 4). The amino acid sequences of the *PsADH1* and *PsADH2* isozymes are 80.5% identical to one another and are 71.9 and 74.7% identical to *S. cerevisiae ADH1*. The *P. stipitis* *ADH* proteins are presumed to be localized in the cytoplasm, as they do not

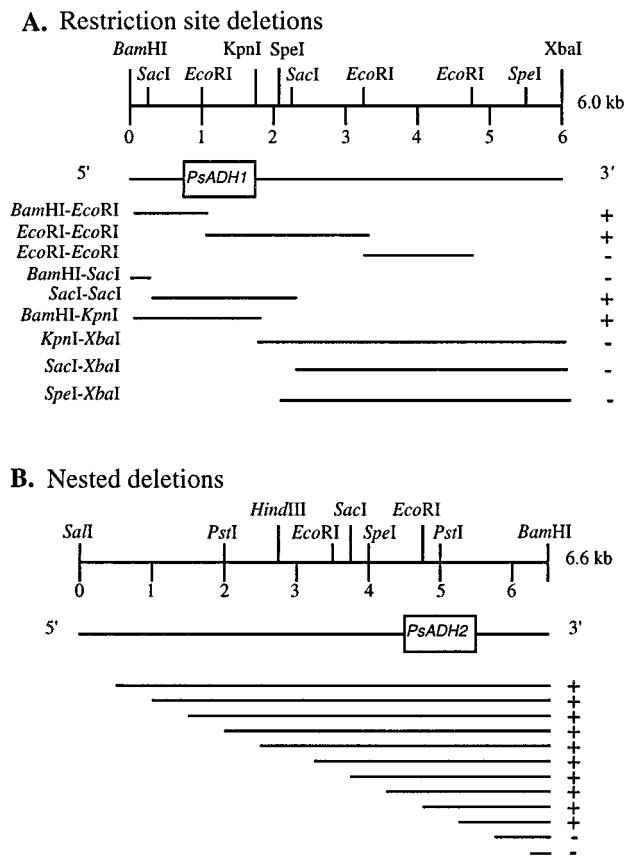


FIG. 2. Southern hybridization to identify genomic regions on clones of *PsADH1* and *PsADH2*. (A) Approximate location of the *PsADH1* coding region on the 6.0-kbp *BamHI-XbaI PsADH1* DNA fragment as determined by restriction mapping and cross-hybridizations of the resulting restriction fragments to *ScADH1*. +, positive hybridization; -, no hybridization. Bold lines represent the fragment containing 5'- and 3'-flanking regions of *PsADH* genes. (B) Approximate location of the *PsADH2* coding region on the 6.6-kbp *BamHI-SalI PsADH2* DNA fragment as determined by nested deletions from the 5' end and cross-hybridizations of the resulting fragments to *ScADH1*. +, positive hybridization; -, no hybridization. The boxes indicate the *PsADH1* (A) and *PsADH2* (B) coding regions.

possess the amino-terminal extension of mitochondrion-targeted ADHs (26, 44, 48).

**Disruption of *PsADH* genes and analysis of disruptant strains.** To examine the physiological role of the two *P. stipitis* ADH isozymes known, we created *Psadh* disruptant strains of *P. stipitis* by the one-step gene replacement technique (29). *P. stipitis ura3* auxotroph PSU1 was transformed with the linear construct carrying the disrupted copy of *Psadh1*, and transformants were selected on glucose medium lacking uracil. The *Ura*<sup>+</sup> transformants were picked randomly and screened by Southern blot analysis (Fig. 5). Genomic DNAs derived from these transformants, parental strain PSU1, and wild-type strain CBS 6054 were digested with *SacI*. Southern blotting of CBS 6054 and PSU1 genomic DNAs with a *PsADH2* probe revealed 2.0- and 3.0-kbp bands for the *PsADH1* and *PsADH2* fragments, respectively (Fig. 5, lanes 1 and 2). Southern blotting of a *Ura*<sup>+</sup> transformant probed with *PsADH2* coding sequences revealed the pattern of fragments expected for a simple gene replacement at *PsADH1* with the *Psadh1::PsURA3* fragment: 3.2- and 3.0-kbp bands were detected for the *SacI* digests (Fig. 5, lane 4). The resulting *Psadh1::PsURA3/Psadh1::PsURA3* ho-

mozygous disruptant was named PSU-218, and its fermentative behavior was tested further.

In the case of the *PsADH2* disruption, *PsLEU2* was inserted into the *PsADH2* coding region (see Materials and Methods). A 4.5-kbp fragment of pJY202 containing the disrupted copy of *Psadh2* was generated by digestion with restriction enzymes *BamHI* and *SacI* and then transformed into the *P. stipitis ura3 Δleu2* auxotroph PLU20 in the presence of restriction enzymes *BamHI* and *SacI*. The resulting transformants were selected for growth in the absence of leucine and screened by PCR by using primers specific for the point at which the *PsLEU2* DNA fragment was inserted into the *PsADH2* gene. One of the transformants selected for growth in the absence of leucine, named PLU-123, showed a 2.1-kbp DNA fragment amplified by PCR, as expected for correct integration (data not shown). Correct homozygous integration of the fragment carrying the disrupted copy of *Psadh2* (*Psadh2::PsLEU2*) into the corresponding genomic locus showed that 3.0-kbp fragments were replaced in the Southern blot analysis by new fragments of increased length because of the insertion of the 2.1-kbp fragments of *PsLEU2* (Fig. 5, lane 5).

We then constructed a strain lacking both *PsADH* genes. PLU-123, a derivative of PLU20 carrying disrupted copies of *Psadh2*, was transformed with the linear fragments which we previously had used to disrupt *PsADH1* in PSU1 and screened by PCR using primers specific for the point at which the *PsURA3* DNA fragment was inserted into the *Psadh1* gene. Two of the initial *Ura*<sup>+</sup> transformants were found to be *Psadh1::PsURA3/Psadh1::PsURA3* and *Psadh2::PsLEU2/Psadh2::PsLEU2* homozygous disruptants. The correct integration of each fragment into the corresponding genomic locus was confirmed by Southern blot analysis: 3.2-kbp (*Psadh1::PsURA3*) and 5.1-kbp (*Psadh2::PsLEU2*) fragments were detected for both *PsADH1* and *PsADH2* gene replacement (Fig. 5, lane 6). This *Psadh* multiple disruptant strain, PLU-1209, was tested along with the previously obtained disruptants for their fermentative and growth properties.

**Fermentation of xylose.** The *Psadh* disruptant strains were compared for appropriate wild-type and parental strains for ethanol production from xylose under oxygen limitation. Compared with those of the control strains, the apparent ethanol production and growth rate of PSU-218 were very low (Fig. 6). At the same time, the level of xylitol production of PSU-218 and double disruptant strain PLU-1209 was much higher than that of the corresponding control strains. On the other hand, disruption of *PsADH2* had little effect on growth rate, ethanol production, or xylitol production. Interestingly, *Psadh* double disruptant strain PLU-1209 failed to grow on xylose but still produced small amounts of ethanol under these conditions.

We then analyzed the ability of the *Psadh* disruptant strains to use ethanol as a sole carbon source under fully aerobic conditions. As can be seen in Fig. 7A, PSU-218 grew as well as CBS 6054 and better than its parent, PSU1, when cultivated in liquid minimal medium (YNB) containing ethanol, while PLU-1209 failed to grow under these conditions. In contrast, PLU-123 grew on ethanol at a rate similar to that of its parental strain, PLU20, indicating that *PsADH2* is dispensable for growth of *P. stipitis* on ethanol unless *PsADH1* is disrupted. PLU-123, a *Psadh2* disruptant, and PLU-1209, a *Psadh1* and *Psadh2* double disruptant, were able to grow on glycerol at rates similar to those of their parental and wild-type strains (Fig. 7B). Growth of the double disruptant on glycerol indicated that its lack of growth on ethanol was not attributable to a loss of respiratory capacity.

Finally, each of the two ADH activities is able to convert ethanol to acetaldehyde and is sufficient to allow cell growth.

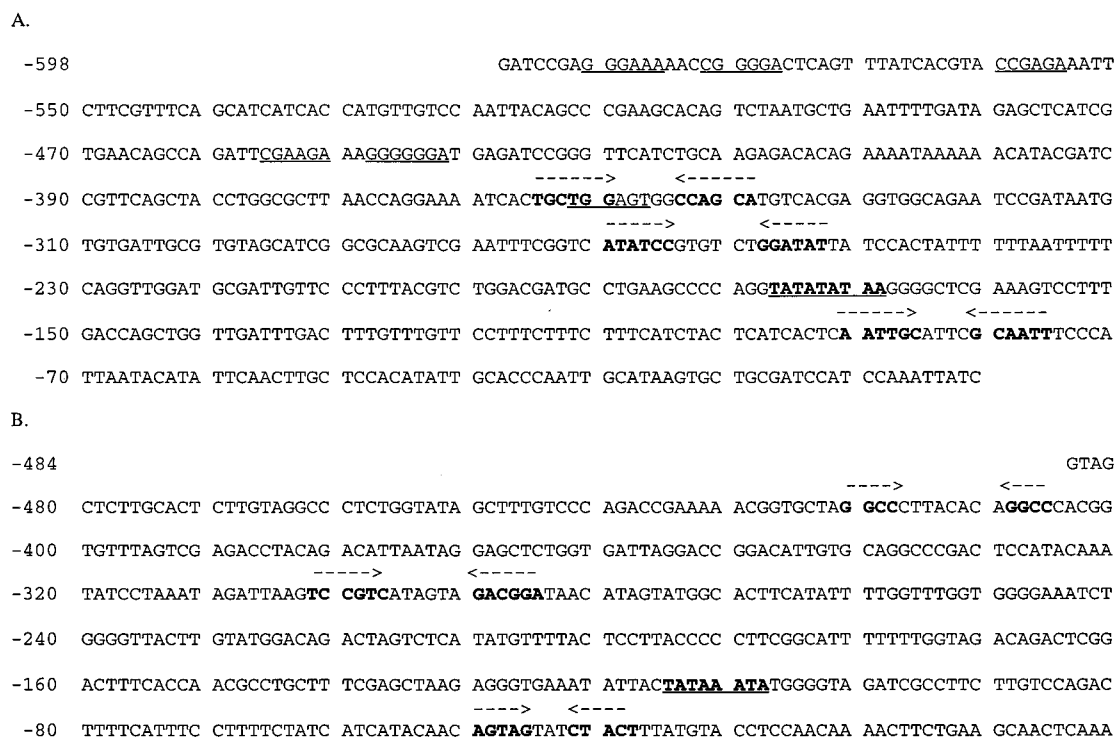


FIG. 3. Upstream sequences of *PsADH* genes and localization of their coding regions. (A) *PsADH1* upstream region showing a putative TATA element (TATATATAA, bold and underlined), hairpins (bold), and repeats (underlined). (B) *PsADH2* upstream region showing a putative TATA element (TATAAATA, bold and underlined) and hairpins (bold).

Moreover, each *Psadh* disruptant strain was still able to accumulate ethanol, although in different amounts (PLU-123 > PSU-218 > PLU-1209) when grown on xylose under oxygen-limited conditions.

## DISCUSSION

The ADH isozymes in *P. stipitis* are encoded by multiple genes. The exact number is unknown. Hybridization of *ScADH2* to the *XbaI* genomic fragments indicated that the *P. stipitis* genome may contain three *ADH* genes. Two fragments, about 9.0 and 23.0 kb in length, strongly cross-hybridized to *ScADH2* (Fig. 1B, lane 8). A third fragment, 4.3 kbp in length, hybridized to a lesser extent. The inability of the *Psadh* double disruptant to grow on ethanol under full aerobiosis implies that no other ADH activities are present under these conditions. The gene for a third isoenzyme may be present but not expressed on ethanol under aerobic conditions.

We cloned and sequenced two structural genes from *P. stipitis*, designated *PsADH1* and *PsADH2*. They both have uninterrupted ORFs of 1,047 nucleotides and show high conservation with respect to other yeast group I ADH proteins. The two ADH isozymes of *P. stipitis* are 80.5% identical at the protein level and 79.5% identical in the coding region at the nucleotide level. *PsADH2* more strongly hybridized to *ScADH1* and *ScADH2* than to *PsADH1* (Fig. 1B, lanes 3, 6, and 9). This may be an artifact of DNA conformation on that fragment, since *P. stipitis* *ADH* genes are structurally closer to one another than to any of the *S. cerevisiae* or *K. lactis* genes. Our designation of the isozymes as *PsADH1* and *PsADH2* is in keeping with the similarity of their physiological roles to those of ADH1 and ADH2 of *S. cerevisiae*. However, they appear to have evolved from one another independently of the divergence between

*ScADH1* and *ScADH2* (Fig. 4). Notably, the deduced amino acid sequences of the *P. stipitis* isozymes showed a conserved amino acid residue (Lys<sup>305</sup>) that is thought to be involved in the substrate-binding cleft. *S. cerevisiae* ADH1 has a methionine in this position, which is thought to contribute to the narrow substrate specificity of this enzyme (38).

Analysis of the 5'-flanking region of *PsADH1* and *PsADH2* revealed consensus sequences for a putative TATA box and an ATG context, which is optimal for highly expressed genes from *S. cerevisiae* (17a). Several direct repeats, including six direct repeats with a slight variant of the consensus sequence A/TGGAGA, and inverted repeats were found in the 5'-flanking region of *PsADH1*. The functional significance of these sequences has not been determined, yet it is tempting to speculate that they represent a recognition site(s) for a *trans*-acting factor(s) involved in the regulation of *PsADH1* expression. Dyad symmetry is typical of the sequences recognized by dimeric DNA-binding proteins containing bacterial helix-turn-helix motifs, and the promoter region implicated in transcriptional regulation of *S. cerevisiae* *ADH2* also contains seven repeats with the consensus sequence A/TGGAGA and a 22-bp sequence with perfect dyad symmetry (2, 39, 49).

Bennetzen and Hall (3) suggested that there is a correlation between the expression level of a gene and its codon usage bias. The codon usage rules derived from *S. cerevisiae* genes seem to apply to *P. stipitis* as well. The *PsADH1* and *PsADH2* genes show a highly biased codon usage very similar to that observed in the corresponding genes of *S. cerevisiae* (data not shown), where only 25 preferred codons are used extensively, whereas the *URA3* gene of *P. stipitis* (47) shows a poorly biased codon usage pattern, as does the same gene of *S. cerevisiae* and *K. lactis*, where it is less highly expressed (40).

Disruption of *PsADH1* and *PsADH2* was more difficult than

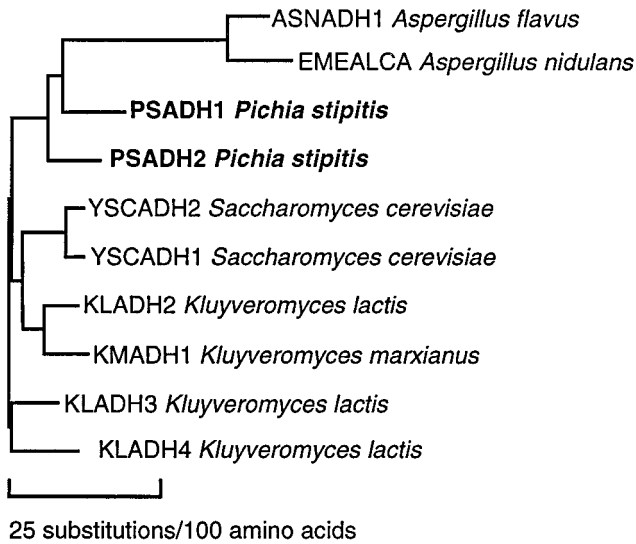


FIG. 4. Phylogenetic tree showing similarity among yeast *ADH* genes. Sequences of 10 different *ADH* genes from the GenBank and EMBL data banks were compared by using the Genetics Computer Group (12) PileUp, Distances, and Grow Tree programs. Source names are shown.

expected. Melake et al. (24) proposed that wild-type strains of *P. stipitis* are haploids. Therefore, disruption should have been easy to accomplish. However, disruption studies occasionally showed what appeared to be heterozygotes, indicating that at least some of the parental strains (or resulting disruptants) are diploids. In contrast to that in *S. cerevisiae*, the frequency of homologous recombination at any given locus in *P. stipitis* seems to be low and varies somewhat from locus to locus within the genome (6). When these disruption studies were carried out, nonhomologous integration of introduced DNA was more common than homologous integration. The basis for the variability and low frequency is not clear. However, several aspects of DNA metabolism, such as DNA repair and transcription, are closely interrelated with homologous recombination (17, 37).

In the case of the *PsADH1* gene disruption, only 1 of 17 *Ura*<sup>+</sup> transformants was a homozygous integrant at the corresponding genomic locus. In addition to this homozygous integration, numerous *URA3* gene conversion, heterozygous, and random-integration events were detected (data not shown). Schiestl and Petes (36) found that introducing the restriction enzyme *Bam*HI together with a *Bam*HI-cut DNA fragment increased the number of transformed cells and showed that the DNA fragment was often integrated into *Bam*HI sites of the host genome. This surprising result led us to explore restriction enzyme-mediated integration in *P. stipitis* for disruption of *PsADH2*. This approach proved successful.

We created *P. stipitis* strains disrupted in each or both structural *ADH* genes. Disruption of *PsADH1* clearly affected the growth rate and ethanol production on xylose under oxygen-limiting conditions. The increase in xylitol production by the *Psadh1* disruptant indicates that in the absence of *PsADH1*, NADH accumulates and favors xylitol production. *PsADH1* appears to be the principal gene responsible for ethanol production because its loss results in slower growth, lower ethanol production, and much greater xylitol production under oxygen-limited conditions, whereas the loss of *PsADH2* has none of these effects. Disruption of *PsADH1* in *P. stipitis* causes this yeast to make even more xylitol than does *Candida shehatae* or

*Pachysolen tannophilus*, where significant xylitol production has been observed under oxygen-limited conditions (15). Xylitol production has been explained by Debus et al. in terms of an electron sink for NADPH, generated in the phosphogluconate pathway (10). On the basis of our present studies, we propose that in *P. stipitis*, xylitol production is normally low because *PsADH1* suppresses the intracellular level of NADH. When *PsADH1* is disrupted, NADH accumulates. Accumulation of NADH would, in turn, shift the equilibrium of the XDH-mediated reaction to favor xylitol over xylulose formation. Fermentative ADH activity is therefore essential not only for ethanol production but also for maintenance of redox balance in the cytoplasm. The accumulation of reducing equivalents in the form of NADH does not seem to be relieved by reoxidation under oxygen-limited conditions or by NADH-linked XR activity. The dependence of xylose metabolism on

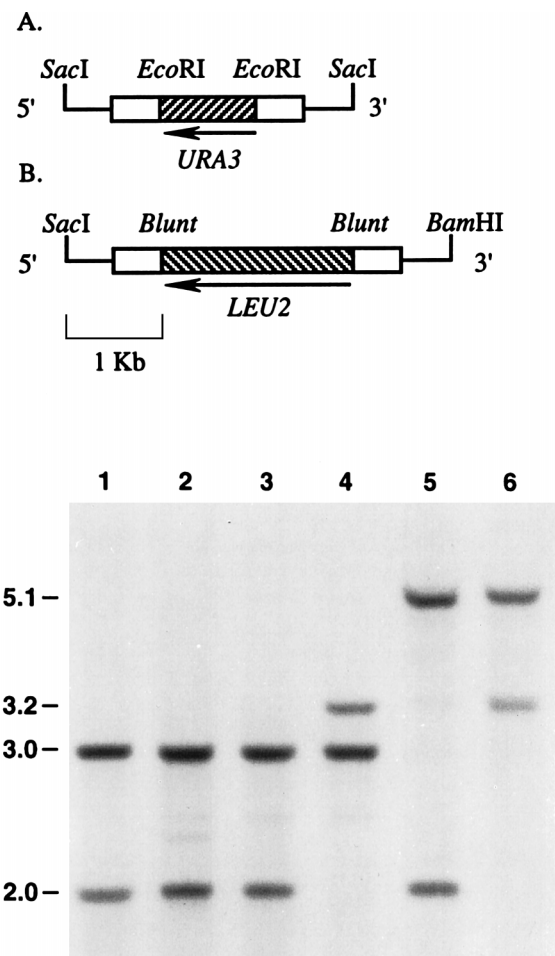


FIG. 5. Construction and Southern blot analysis confirming replacement of *PsADH* genes. Linear fragments carrying disrupted copies of the *PsADH1* (pJY102) (A) and *PsADH2* (pJY202) (B) genes were constructed and introduced into the genome as described in the text. These disruption constructs contain approximately 1.0 kbp of 5'- and 3'-flanking *PsADH1* (A) and *PsADH2* (B) homology. Bold lines represent fragments containing 5'- and 3'-flanking regions used for gene replacement. White boxes indicate functional *LEU2* (A) and *URA3* (B) genes from *P. stipitis*. Southern hybridization results are shown at the bottom. Strain CBS 6054 (lane 1) is the *P. stipitis* wild type. *P. stipitis* PSU1 (lane 2) and PLU20 (lane 3) are parental strains used for *PsADH1* and *PsADH2* gene replacements, respectively. PSU218 (lane 4) and PLU123 (lane 5) are strains disrupted in *PsADH1* and *PsADH2*, respectively. PLU1209 (lane 6) is disrupted in both *PsADH1* and *PsADH2*.

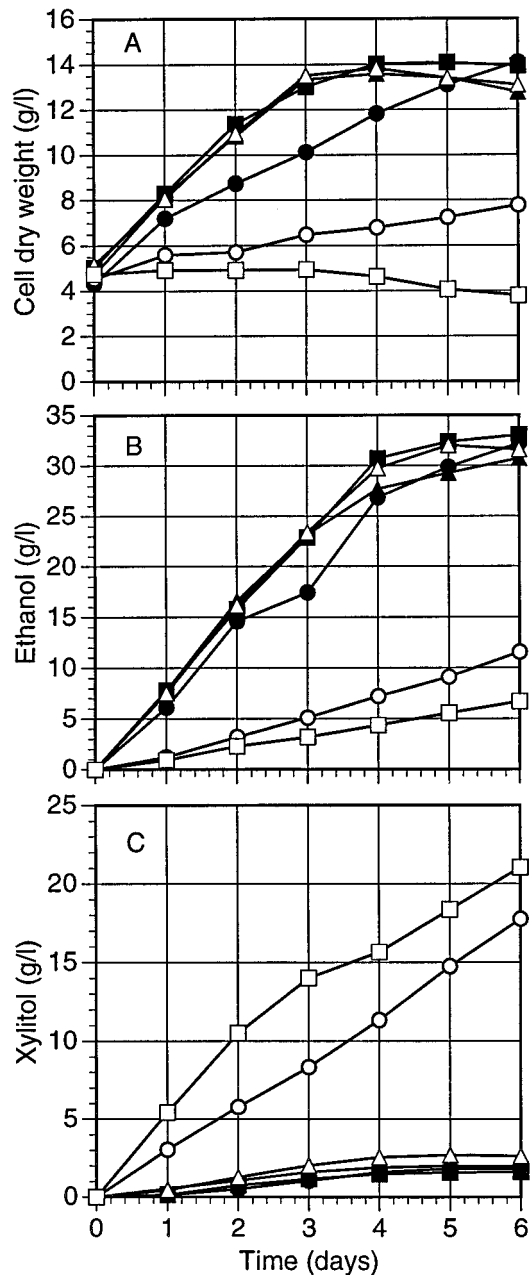


FIG. 6. Cell growth (A), ethanol production (B), and xylitol production (C) of wild-type, parental, and disruptant strains of *P. stipitis* under fermentative conditions. Inocula were grown in flasks for 48 h, and initial cell densities of each strain were adjusted to be similar. Each value represents the mean of two determinations. Symbols: ■, CBS 6054 (wild type); ●, PSU1; ▲, PLU20; △, PLU123 (*Psadh2* disruptant); ○, PSU218 (*Psadh1* disruptant); □, PLU1209 (*Psadh* double disruptant).

NADH recycling by PsADH1 could be why relatively slow growth was observed with *Psadh1* strains of *P. stipitis* (PSU-218 and PLU-1209) under oxygen-limited conditions. *Psadh1* strains still take up xylose, grow, and accumulate ethanol—albeit to a lesser extent than the parent under oxygen-limited conditions. On the other hand, the *Psadh* double-disruptant strain failed to grow on xylose and produced even less ethanol and more xylitol under these conditions. These results suggest that the PsADH2 isozyme is also involved in xylose fermenta-

tion and growth, although it probably plays a minor role compared to PsADH1.

The analysis of the *Psadh* disruptant strains, which contained either one or the other of the residual ADH activities, showed that all were able to grow aerobically on media containing ethanol as a sole carbon source. This result suggests that either PsADH1 or PsADH2 can confer the ability to oxidize ethanol. PSU-218 (*Psadh1::PsURA3*) actually grew better on ethanol than did the parental or wild-type strain, implying that *PsADH2* might be overexpressed and more important under these conditions. Because disruption of *PsADH2* had no discernible effect on fermentation or growth on xylose, an open question is whether *PsADH2* is expressed only when *PsADH1* is disrupted or whether it plays some other role.

The simultaneous presence of ethanol-producing and ethanol-oxidizing activities in the cytoplasm could result in a futile cycle. This could be why KIADH4 is compartmentalized within mitochondria in *K. lactis* (23) and catabolite repression of *ADH2* occurs in *S. cerevisiae* (30). Oxygen-dependent regulation of *PsADH* expression could avoid futile cycling in the cytoplasm of *P. stipitis* if respirative enzyme activities were repressed and fermentative enzyme activities were induced under oxygen-limited conditions. In *P. stipitis*, a Crabtree-neg-

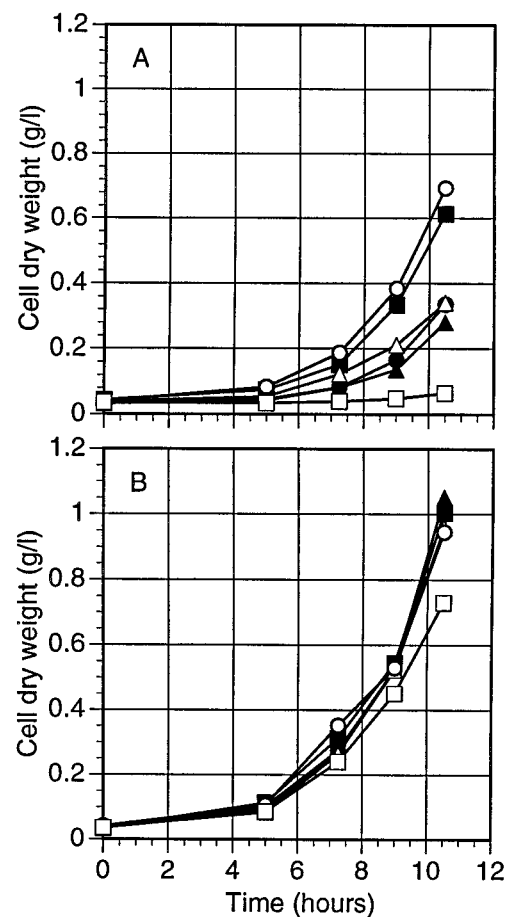


FIG. 7. Aerobic growth of *P. stipitis* wild-type CBS 6054, parental, and *Psadh* disruptant strains on either ethanol (A) or glycerol (B). Cells were grown in YNB with either 2% ethanol or 3% glycerol plus 100  $\mu$ g of leucine or uridine/ml, as needed, with shaking in a 125-ml baffled flask at 180 rpm and 30°C. Symbols: ■, CBS 6054 (wild type); ●, PSU1 (*ura3-2/ura3-2*); ▲, PLU20 (*ura3-3/ura303 Psleu2Δ-1/Psleu2Δ-1*); △, PLU123 (*Psadh2* disruptant); ○, PSU218 (*Psadh1* disruptant); □, PLU1209 (*Psadh* double disruptant).

ative yeast, fermentation enzymes such as ADH and PDC are induced only under oxygen limitation (25), and no fermentation occurs on medium containing 3% xylose under fully aerobic conditions (7). This is in keeping with a hypothesis of oxygen-dependent regulation of *ADH* expression in *P. stipitis*, as discussed above. The different behavior of these yeasts compared to that of *S. cerevisiae* is most probably explained by differences in the nature of the fundamental mechanisms by which fermentation is regulated.

The *Psadh* double-disruptant strains are not able to utilize ethanol, indicating that no other important ADH activities are present in *P. stipitis* when it is growing on ethanol under aerobic conditions. However, the *Psadh* double disruptants still produce ethanol from xylose, implying that a third enzyme might be expressed under oxygen-limited conditions. ADH null strains of both *S. cerevisiae* (14) and *K. lactis* (31) can still produce residual amounts of ethanol when grown aerobically on 2% glucose. The biochemical basis of this phenomenon remains unclear. In any case, the addition of a respiratory inhibitor such as 2,4-dinitrophenol, sodium azide, or potassium cyanide should immediately block the production of ethanol in the *Psadh* null strain because unimpaired mitochondrial function is necessary for growth on and alcoholic fermentation of xylose in *P. stipitis* (20). The function of this respiratory activity is not clear, but it could be necessary to generate energy for xylose transport (19). Experiments are under way to unravel the molecular basis for the expression of *PsADH* genes in this yeast.

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