YEAST VOL. 11: 839–847 (1995)

A Short-Chain Dehydrogenase Gene from *Pichia stipitis* Having D-Arabinitol Dehydrogenase Activity

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Received 19 January 1995; accepted 15 March 1995

An NAD^+-dependent D-arabinitol dehydrogenase (polyol dehydrogenase) gene was isolated from *Pichia stipitis* CBS 6054 and cloned in *Saccharomyces cerevisiae*. The gene was isolated by screening of a λ-cDNA library with a zymogram technique. D-Arabinitol, xylitol, D-glucitol and galactitol are substrates for the recominant protein. With D-arabinitol as substrate the reaction product is D-ribulose. The molecular weight of the native tetramer enzyme is 110 000 Da and the monomer is 30 000 Da. The amino acid sequence is homologous to the short-chain dehydrogenase family. It is 85·5% identical to a D-arabinitol dehydrogenase from *Candida albicans*. The gene in *P. stipitis* was induced by D-arabinitol and *P. stipitis* was able to grow on D-arabinitol. The physiological role of D-arabinitol metabolism is discussed.

KEY WORDS — yeast; *Pichia stipitis*; *Saccharomyces cerevisiae*; overexpression; *ARDH*; D-arabinitol dehydrogenase; zymogram screening; arabinitol metabolism; xylose metabolism

INTRODUCTION

Acyclic polyols like glycerol, D-arabinitol, D-mannitol, xylitol and erythritol are common in nature. As a response to high salinity or high sugar concentrations, some fungi and yeast accumulate intracellular glycerol and D-arabinitol (Pfyffer and Rast, 1989; Tokuoka *et al.*, 1992). *Zygosaccharomyces rouxii*, an osmophilic yeast, accumulates elevated levels of intracellular D-arabinitol when grown on high sugar concentrations (Tokuoka *et al.*, 1992). *Candida tropicalis* produces D-arabinitol when grown on *n*-alkane. Up to 50% of the consumed *n*-alkane was converted to D-arabinitol (Hattori and Suzuki, 1974). The human pathogen *Candida albicans* produces D-arabinitol during infection, so that elevated levels of D-arabinitol are detected in the body fluid of humans with candidosis (Deacon, 1986).

Several micro-organisms are able to metabolize D-arabinitol as the sole carbon source. The first enzymatic step of the metabolism of D-arabinitol is an oxidation reaction by a D-arabinitol-specifc polyol dehydrogenase. D-Arabinitol dehydrogenases (ArDH) characterized from fungi and yeast oxidize D-arabinitol to D-ribulose (Wong *et al.*, 1993; Quong *et al.*, 1993), whereas bacterial ArDH oxidizes D-arabinitol to D-xylulose (Charnetzky and Mortlock, 1974).

Here we present the isolation of a D-arabinitol-specific polyol dehydrogenase (*ARDH*) encoding gene from *Pichia stipitis*, the over-expression of the gene in *Saccharomyces cerevisiae* and the enzymatic properties of the corresponding enzyme. In addition to this polyol dehydrogenase, which is a member of the short-chain dehydrogenase family (Persson *et al.*, 1994), *P. stipitis* also has another polyol dehydrogenase, belonging to the medium-chain alcohol dehydrogenase family (Persson *et al.*, 1994). Both enzymes have xylitol and D-glucitol (sorbitol) as common substrates. The present study shows that the former has highest activity with D-arabinitol, whereas the latter has highest activity with xylitol (Rizzi *et al.*, 1989). Thus, polyol dehydrogenases from two different families with overlapping activities are present in *P. stipitis* (Persson *et al.*, 1994).

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CCC 0749–503X/95/090839–09

MATERIALS AND METHODS

Plasmids and strains

A *P. stipitis* CBS 6054 λ-gt11 cDNA library (Hallborn *et al.*, 1991) was used for zymogram screening. The pSP72 vector (Promega, Madison, WI, U.S.A.) was used for subcloning and sequencing of the *ARDH* gene. The cosmid p3030 (Penttilä *et al.*, 1984) was used for the construction of a genomic library from *P. stipitis*. The yeast expression vector pMA 91 (Mellor *et al.*, 1983) containing the PGK promoter and terminator was used for expression of the *ARDH* gene, giving the pARA100 vector. *Escherichia coli* strain DH5α [F^-, φ80 d*lac* ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo* R *rec* A1 *end* A1 *hsd* R17(r_k^-, m_k^+) *sup* E44λ^- *thi*-1 *gyr* A96 *rel* A1] (Gibco BRL, Gaithersburg, MD, U.S.A.) was used as cloning host and *E. coli* HB101 [*sup* E44 *hsd* S20 (r_b^- m_b^-) *rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rsp*L20 *xyl*-5 *mtl*-1 λ^-] (Promega, Madison, WI, U.S.A.) was used as host for the cosmid library. Yeast transformants were obtained by transforming the strain H158 (*S. cerevisiae* GPY55-15Ba: *leu*2-3, *leu*2-112, *ura*3-52, *trp*1-289, *his*4-519, *prb*1, cir^+ obtained from Gregg Payne, Berkeley University, California) with the pARA100 vector, giving the strain *S. cerevisiae* S700.

Transformation of E. coli *and* S. cerevisiae

E. coli DH5α was transformed by electrotransformation using the Bio-Rad electroporation equipment (Bio-Rad Laboratories, Richmond, U.S.A.), according to the 'High efficiency electrotransformation of *E. coli* method by Bio-Rad. Transformants were selected on LB-ampicillin plates. *S. cerevisiae* was transformed using the spheroplast method (Hinnen *et al.*, 1978). Transformants were selected on SC-leu plates (Sherman *et al.*, 1983).

Zymogram staining

Staining of native gradient PAGE gels and plaques from the cDNA library on nitro-cellulose membranes was performed with a zymogram staining solution containing 0·1 M-phosphate buffer pH 7·0, 0·4 M-polyol (D-arabinitol, xylitol, D-glucitol, ribitol, or L-arabinitol), 1·5 mM-NAD^+, 0·25 mM-nitroblue tetrazolium, and 0·06 mM-phenazine metosulphate. The staining was performed at room temperature for 30 min to 2 h depending on protein concentration and polyol used. The zymogram staining seemed to be more stable with a phosphate buffer at pH 7 than with a Tris buffer at pH 9.

Screening of cDNA library by the zymogram technique

Replicas of the cDNA library on nitro-cellulose membranes incubated overnight in 10 mM-IPTG at 37°C were screened by the zymogram technique.

Screening of cosmid library

A genomic library of *P. stipitis* was constructed using the cosmid p3030. Genomic DNA was partially digested by *Sau*3A and the fragments were separated on a 10–40% continuous sucrose gradient. Fractions containing 15–25 kb fragments were pooled and used for ligation of *Bam*HI-cleaved cosmid p3030. The ligation mixture was packaged *in vitro* into λ-phage particles (Amersham, Bucks, U.K.) and used for infection of *E. coli* HB101. The cosmid library in *E. coli*, plated on LB-ampicillin plates, was screened by colony hybridization (Sambrook *et al.*, 1989) on replica filters (Millipore HA, pore size 0·45 μm), using a PCR-amplified, random-primed labelled [α-^{32}P] cDNA probe (Boehringer, Mannheim, Germany). Positive clones were verified by sequencing.

PCR amplification

The cDNA fragment having polyol dehydrogenase activity was amplified by PCR (Güssow and Clacksow, 1989) using vector-specific primers. The amplified sequence was cut with *Bam*HI and cloned into the pSP72 vector at the *Bam*HI site for sequencing. A radioactive probe obtained by PCR amplification using internal primers and radiolabelled dATP (S^{35}) was used to isolate a chromosomal sequence from a *P. stipitis* cosmid library. The coding region was amplified by PCR using primers corresponding to the 5′ (5′-GGA TCC AGA TCT ATG GAC TAC TCA TAC GCT) and 3′ (5′-GGA TCC AGA TCT TTA AAC TGT GGG TCG TAT) end of the gene, respectively. The primers had non-complementary 5′ ends carrying *Bam*HI and *Bgl*II restriction sites for subcloning. The PCR reaction mixture (100 μl) contained 0·1 μM forward and reversed primer, 50 μM-dNTP, 1×10^9 molecules of template and 2 U of Taq DNA polymerase (Boehringer GmbH, Mannheim, Germany) in standard PCR buffer (Boehringer). The Perkin-Elmer Cetus DNA Thermal Cycler 480 was used for PCR amplification. The first PCR cycle was performed with a

three-step cycle (30 s at 95°C [denaturation], 30 s at 45°C [primer annealing] and 60 s at 72°C [polymerization]) the other 25 amplification cycles were performed as a two-step PCR amplification reaction (30 s at 95°C [denaturation] and 60 s at 72°C [primer annealing and polymerization]) in order to increase the accuracy of the amplification.

Sequencing and computer analysis

PCR-amplified cDNA and genomic DNA was sequenced (the dideoxy method) from both strands using internal oligonucleotide primers (Zagursky *et al.*, 1986). The nucleotide sequence and the corresponding amino acid sequence of the structural gene were analysed using the GCG sequence analysis software package (Genetics Computer Group, Inc., Madison, Wisconsin, U.S.A., 1992, Version 7.1).

Nucleotide sequence accession number

The cDNA sequence of the *ARDH* gene has been deposited in the EMBL database under the accession number Z46866.

Cultivation of transformant

S. cerevisiae S700 and H158 were cultivated in minimal medium containing 6·7 g/l yeast nitrogen base without amino acids (YNB-AA) and 0·05 g/l L-tryptophan, L-histidine and uracil (Sc-leu medium). *P. stipitis* was cultivated in 0·3% yeast extract (Difco), 0·3% malt extract (Difco), 0·5% peptone (Difco), 1·9% KH_2PO_4, 0·3% $(NH_4)_2HPO_4$, 0·1% $MgSO_4$ (YMP medium) and 2% carbon source (D-glucose, D-xylose or D-arabinitol), pH 5. Inocula were grown aerobically overnight in 1-l shake flasks containing 50 ml of either Sc-leu medium with 20 g/l glucose (*S. cerevisiae*) or YMP with 2% D-glucose, D-xylose or D-arabinitol (*P. stipitis*). A pre-culture of 500 ml medium was inoculated and grown for 12 h. The cells were harvested, washed and inoculated to the batch cultivation, giving an initial cell mass concentration (dry weight) of 0·3–0·6 g/l. Batch cultivations were performed in 1-l shake flasks containing 250 ml medium. Cells were harvested in the late exponential growth phase.

Preparation of cell-free extract

10 g wet-weight of cells were suspended in a total volume of 25 ml disintegration buffer (0·1 M-phosphate buffer, 5 mM-EDTA, 5 mM-2-mercaptoethanol, pH 7.0) and freeze-pressed twice with an X-press (Biox, Göteborg, Sweden). Cell-debris was removed by centrifugation (15 000 *g*, 15 min).

Enzyme assays

Polyol dehydrogenase activity was measured spectrophotometrically by following the reduction of NAD^+ or $NADP^+$ at 340 nm (Smiley and Bolen, 1982). When different polyols were used, the assay mixture contained 0·1 M-Tris buffer pH 9·0, 10 mM-NAD^+ (Sigma, St Louis, MO, U.S.A.), 0·1 M-sugar alcohol (Sigma) and 0·05 ml sample. For Km_{NAD^+} measurements, the NAD^+ concentration was varied between 0·1–10 mM. For $Km_{D\text{-arabinitol}}$ measurements, the D-arabinitol concentration was varied between 10–400 mM. Ketose reductase activity (D-xylulose and D-ribulose) was measured by following the oxidation of 0·34 mM-NADH in 0·1 M-Tris buffer, pH 7·0, holding 1·5 mM-ketose, and 0·05 ml sample. Sample concentration was adjusted to give changes in absorbancy between 0·05 and 0·15 ΔE per min. Enzyme units are defined as μmol cofactor reduced or oxidized per min at 30°C. Specific activities are defined as units per mg protein.

Protein determination

Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as standard.

Gel electrophoresis

Native gradient PAGE (4–20% T, 2·6% C) (Mini-PROTEAN II, Bio-Rad Laboratories, Richmond, U.S.A.) was run in Tris glycine buffer, pH 8·3, at 150 V for 3 h. High molecular weight (HMW) protein standard (Pharmacia Fine Chemicals, Uppsala, Sweden) was used as reference. After electrophoresis the gel was immersed in 0·1 M-phosphate buffer, pH 7·0 for 30 min. Samples were stained with the zymogram technique using different polyols as substrate. The HMW standard was stained with the Coomassie method (0·1% Coomassie blue R-250, 40% methanol, and 10% acetic acid).

SDS-gradient PAGE (4–20% T, 2·6% C); Mini-PROTEAN II, Bio-Rad Laboratories, Richmond, U.S.A.) was run in Tris glycine buffer, pH 8·3, containing 0·1% SDS, at 200 V for 1 h. Low molecular weight (LMW) protein standard (Pharmacia Fine Chemicals, Uppsala, Sweden) was used as

reference. After electrophoresis the gel was stained with the Coomassie method (see above).

Analysis of in vitro *activity product by HPLC*

From *in vitro* activity assays containing 0·1 M-Tris buffer, pH 9·0, 0·1 M-D-arabinitol, 10 mM-NAD^+, and cell-free extract diluted 2000 times, an aliquot was withdrawn when the reaction was at equilibrium (as a reference an aliquot was withdrawn before the addition of cell-free extract). The samples were analysed by HPLC (Varian™) using an Aminex ion exclusion HPX-87H cation exchange column at 80°C with water as mobile phase at a flow rate of 0·6 ml/min. The detection of compounds was obtained from a refractive index detector (Waters 410™ differential refractometer). Standards used were D-arabinitol, xylitol, D-xylulose and D-ribulose (analytical grade, Sigma, St Louis, MO, U.S.A.).

RESULTS

Cloning of the ARDH *gene*

An expression cDNA library (λ-gt11) of *P. stipitis* CBS 6054 (Hallborn *et al.*, 1991) was screened for xylitol dehydrogenase activity from plaque lifts on nitro-cellulose membranes (zymogram staining). Two positive clones were isolated and PCR amplified using primers specific to the cloning site of the λ-vector. Restriction enzyme analysis with *Eco*RI of PCR-amplified DNA showed that the two clones were of the same size and had identical restriction fragment patterns. One of the PCR-amplified cDNA clones was subcloned into pSP72 and sequenced. The origin of the cloned sequence was verified by screening a genomic library of *P. stipitis* with a radioactive probe of the cDNA sequence. The genomic clone was subcloned by PCR amplification into pSP72 and the yeast expression vector pMA91, giving the pARA100 vector.

Sequence analysis

In Figure 1 the nucleotide sequence of the cloned cDNA of the *ARDH* gene is shown. The total length of the isolated cDNA sequence is 1157 bp, with a 5′ non-coding region of 190 bp and a 3′ non-coding region of 130 bp. As in many highly expressed yeast genes, an A is located at the −3 position relative to the ATG start codon. The nucleotide sequence at the ATG site (position −9 to 3) has a 12 bp sequence (TATACTACAATG)

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   1  AAATCATAGAATAGAGAATCCCAATAGCACAAGCTCCTGATTTCCTTGCATTATTTGCCC   60
  61  GCGCTCAAGTATTTTTTAGACTGAGTCTTCCATACTTCGTACTGATATAAACAAATATTG  120
 121  CACAAACTACTACTAGTGAATTACTAATAGTTTCCACCAAATACAGCCACTACACACTAC  180
 181  ATATACTAACATGGACTACTCATACGCTAACGTTGTTCCCAACTTCAGATTGGACGGAAG  240
                MetAspTyrSerTyrAlaAsnValValProAsnPheArgLeuAspGlyAr
 241  ATTGGCTATTATTACCGGAGGTTCTGGTGGTTTGGCCGCAGTCATTTCGCGTGCCTTGTT  300
      gLeuAlaIleIleThrGlyGlySerGlyGlyLeuAlaAlaValIleSerArgAlaLeuLe
 301  GGCCCAGGGCGCTGATGTTGCTCTCATTGACATGAACTTGGAAAGAACCAAGTCCGCTGC  360
      uAlaGlnGlyAlaAspValAlaLeuIleAspMetAsnLeuGluArgThrLysSerAlaAl
 361  CAAAGAAGTTTTGGGCTGGGGTGAAGAGACGTTGAAGGGTGAACACGCTTCAGCCATCGG  420
      aLysGluValLeuGlyTrpGlyGluGluThrLeuLysGlyGluHisAlaSerAlaIleGl
 421  CCAAGTTTCCGCCTGGTCCTGCAACATTGGGGATGCTGAGGCAGTAGACGCTACTTTCAG  480
      yGlnValSerAlaTrpSerCysAsnIleGlyAspAlaGluAlaValAspAlaThrPheSe
 481  CTCCATCAACGAACACCACGGCAAGATCGCTGACTTGTTGATTAACACCGCTGGATACTG  540
      rSerIleAsnGluHisHisGlyLysIleAlaAspLeuLeuIleAsnThrAlaGlyTyrCy
 541  TGAAAACTTCCCTGCCGAAACGTACCCGGCTACTAACGCTGAAAGCATCATGAAGGTGAA  600
      sGluAsnPheProAlaGluThrTyrProAlaThrAsnAlaGluSerIleMetLysValAs
 601  CGGTTTGGGCTCATTCTACGTTTCGCAATCGTTCGCTAGACCATTGATCCAGAACAACTT  660
      nGlyLeuGlySerPheTyrValSerGlnSerPheAlaArgProLeuIleGlnAsnAsnLe
 661  GAGAGGCTCTATCATCTTGATTGGCTCAATGTCTGGAACAATTGTCAACGACCCACAACC  720
      uArgGlySerIleIleLeuIleGlySerMetSerGlyThrIleValAsnAspProGlnPr
 721  CCAATGTATGTACAACATGTCCAAGGCTGGAGTGATCCACTTGGTCAGATCGTTGGCCTG  780
      oGlnCysMetTyrAsnMetSerLysAlaGlyValIleHisLeuValArgSerLeuAlaCy
 781  CGAATGGGCCAAGTACAACATCAGAGTCAACACCTTATCACCAGGCTATATTTTGACTCC  840
      sGluTrpAlaLysTyrAsnIleArgValAsnThrLeuSerProGlyTyrIleLeuThrPr
 841  TTTAACCAGAAACGTGATTTCTGGCCACACAGAGATGAAGGAAGCCTGGGAATCCAAGAT  900
      oLeuThrArgAsnValIleSerGlyHisThrGluMetLysGluAlaTrpGluSerLysIl
 901  CCCCATGAAGAGAATGGCCGAACCCAAGGAATTCGTGGGGTCCATCTTATACTTGGCAAG  960
      eProMetLysArgMetAlaGluProLysGluPheValGlySerIleLeuTyrLeuAlaSe
 961  CGAGACTGCTTCTTCCTACACTACGGGCCACAATTTGGTTGTGGACGGAGGATATGAATG 1020
      rGluThrAlaSerSerTyrThrThrGlyHisAsnLeuValValAspGlyGlyTyrGluCy
1021  CTGGTAGATACCGCTCCCATTTTAATGAAACGACCACGATACGACCCACAGTTTAATTTC 1080
      sTrpEnd
1081  ACAAGTATAAAGTTTATATGTCGCTACTGTTTTATAGAGAAAGCCAAAATTGACGAAACA 1140
1141  TAAACAAAAAAAAAAAA 1157
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Figure 1. The cDNA sequence of the *ARDH* gene and the deduced amino acid sequence of the ArDH enzyme from *Pichia stipitis*. A 12 bp region that is identical to the *XYL1* gene of *P. stipitis* at the ATG start site is underlined.

identical to the ATG site of the *XYL1* gene from *P. stipitis* (Hallborn *et al.*, 1991). The structural gene is 837 bp long, starting from the first ATG of the cDNA and ending with a TAG stop codon. The sequence of the genomic gene is the same as the cDNA sequence, thus no introns are present within the gene. The codon usage within the open reading frame is similar to *S. cerevisiae* highly to moderately expressed genes, with a codon bias index of 0·3. The corresponding polypeptide is 278 amino acids long with a deduced molecular weight of 30 000 Da. Homology search in the EMBL and GenBank databases using the GCG computer program TFASTA gave 85·5% identity to a D-arabinitol dehydrogenase from *C. albicans* (Wong *et al.*, 1993) and up to 25–28% identity to other polypeptides of the short-chain dehydrogenase family. The nucleotide sequence is 76% identical to the *ARDH* gene from *C. albicans* throughout the whole sequence (Figure 2). The *C. albicans* gene has 9 bp more (three amino acids)

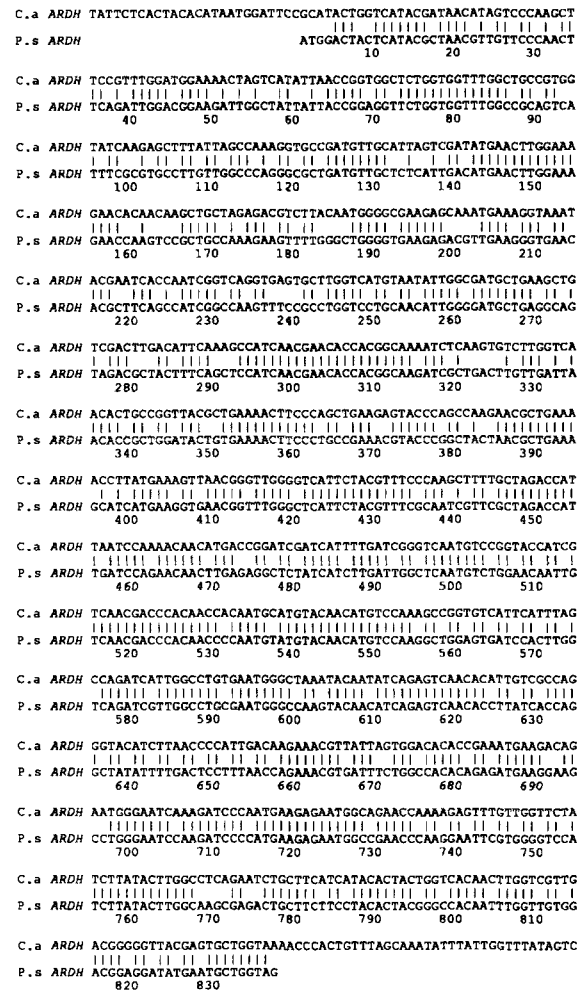

Figure 2. Alignment of *ARDH* genes from *Candida albicans* and *Pichia stipitis* using the GCG program Fasta, showing 76·0% identity in 832 bp overlap.

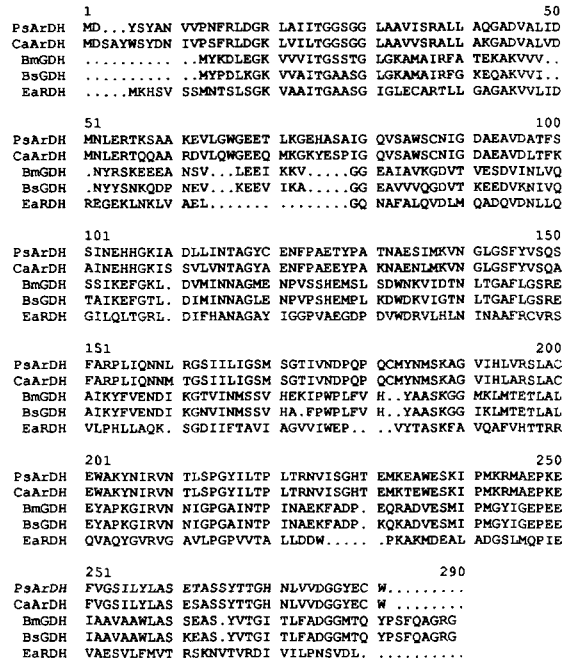

Figure 3. Alignment of *P. stipitis* D-arabinitol dehydrogenase (PsArDH) with *C. albicans* D-arabinitol dehydrogenase (CaArDH), *B. megaterium* glucose dehydrogenase (BmGDH), *B. subtilis* glucose dehydrogenase (BsGDH) and *E. aerogenes* ribitol dehydrogenase (EaRDH). Protein sequences were compared and overlapped in a multiple sequence format with the GCG program Pileup.

inserted after the sixth bp compared with the *P. stipitis* gene (Figure 2). Alignment of the ArDH to polypeptides of the short-chain dehydrogenase family (Figure 3) shows the conserved amino acids of the family. In this family the nucleotide binding domain (βαβ structure) is in the N-terminal part of the polypeptide (Eklund *et al.*, 1976; Jörnvall *et al.*, 1981, 1984). In all polypeptides except ArDH the motif of the highly conserved glycines in the nucleotide binding domain are: Gly-Xxx-Xxx-Xxx-Gly-Xxx-Gly (Jörnvall *et al.*, 1984; Ghosh *et al.*, 1991). In ArDH the last glycine is replaced by an alanine. In the substrate binding domain (the C-terminal part), the amino acid residuces tyrosine (Tyr 181) and lysine (Lys 185) are highly conserved within the family (Persson *et al.*, 1994).

Expression of the ARDH *gene in* S. cerevisiae

Transformants of *S. cerevisiae* H158 containing the pARA100 plasmid were isolated (*S. cerevisiae* S700) and cultivated in Sc-leu medium in order to measure *in vitro* polyol dehydrogenase activities. Enzymatic activities were measured from cell-free extracts. Purification of ArDH prior to measurement was not done since *S. cerevisiae* has no or very low activities of different polyol dehydrogenases (Barnett, 1968) and the cloned *ARDH* gene was overexpressed. In Table 1 specific activities in cell-free extracts with different polyols are summarized. The highest specific activity was obtained with D-arabinitol as substrate (68·25 μmol min^{-1} per mg protein). $Km_{D\text{-arabinitol}}$ and Km_{NAD^+} values were determined to 20 mM and 0·2 mM, respectively. The enzyme had high activities also with xylitol, D-glucitol and galactitol (5·4, 4·0 and 1·8 μmol min^{-1} per mg protein, respectively).

Table 1. Polyol dehydrogenase activities in *S. cerevisiae* S700 and *P. stipitis* CBS 6054.

	S. cerevisiae S700		*P. stipitis* CBS 6054					
	D-Glucose grown		D-Arabinitol grown		D-Xylose grown		D-Glucose grown	
Substrate	Spec. act.	Rel. act.	Spec. act.	Rel. act.	Spec. act.	Rel. act.	Spec. act.	Rel. act.
D-Arabinitol	68·25	100	0·78	85·7	ND*	0	ND	0
Xylitol	5·40	7·9	0·91	100	3·44	100	0·10	100
D-Glucitol	1·79	2·6	0·48	52·7	1·21	35·2	0·04	40·0
Ribitol	0·13	0·2	0·46	50·5	1·61	46·8	0·03	30·0
L-Arabinitol	ND	0	0·01	1·1	0·04	1·2	0·03	30·0
Galactitol	3·97	5·6	—	—	—	—	—	—
D-Mannitol	ND	0	0·58	63·7	ND	0	0·10	100
Glycerol	ND	0	—	—	—	—	—	—
Ethanol	ND	0	—	—	—	—	—	—

Spec. act., specific activity (U mg^{-1} protein); Rel. act., relative activity (% of the highest activity). ND, Not detectable. *No D-arabinitol dehydrogenase activity was detected for *P. stipitis* grown on xylose even though the gene was isolated from xylose-grown cells.

$Km_{xylitol}$ was determined to 300–400 mM. Addition of 10 mM-EDTA to the assay mixture resulted in no significant reduction of activity, thus no divalent cations are required for activity. The product from the enzymatic conversion of D-arabinitol was identified as ribulose by HPLC analysis. Reversed activities with NADH and either D-ribulose or D-xylulose as substrates were 14·8 μmol min^{-1} per mg protein and 0·2 μmol min^{-1} per mg protein, respectively. No measurable activity was detected with NADPH as cofactor.

PAGE analysis

SDS–PAGE analysis of the cell-free extract of *S. cerevisiae* S700 showed that the recombinant protein produced by the transformant was of the same size as that deduced from the *ARDH* gene sequence and that the protein was the major intracellular protein expressed (Figure 4A). Separation of cell-free extract on native-gradient PAGE, stained with the zymogram technique, showed one dense band with an estimated molecular weight of 110 000 Da. Thus the functional enzyme is a tetramer (Figure 4B).

Growth studies of P. stipitis *and* S. cerevisiae *S700 on plates containing arabinose and arabitol*

The relative growth rate of *P. stipitis* on plates containing D-arabinitol was compared with plates containing either D-arabinose, L-arabitol, L-arabinose, xylitol or D-xylose (Figure 5). Plates containing either D-glucose or no carbon source

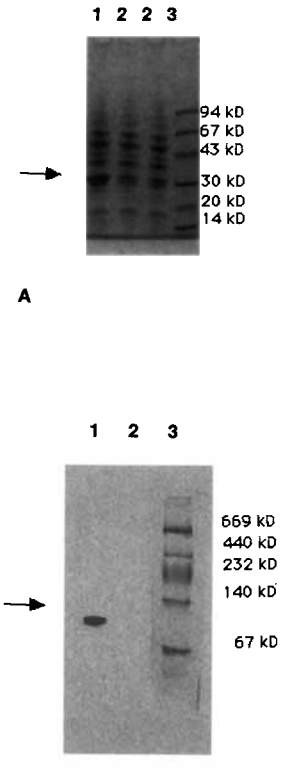

Figure 4. ArDH produced by *S. cerevisiae* S700 analysed by Coomassie staining of SDS–PAGE (A) and by zymogram staining of native gradient PAGE (B). Lane 1, cell-free extract of *S. cerevisiae* S700. Lane 2, cell-free extract of reference strain. Lane 3, molecular weight markers. The arrows mark the position of ArDH.

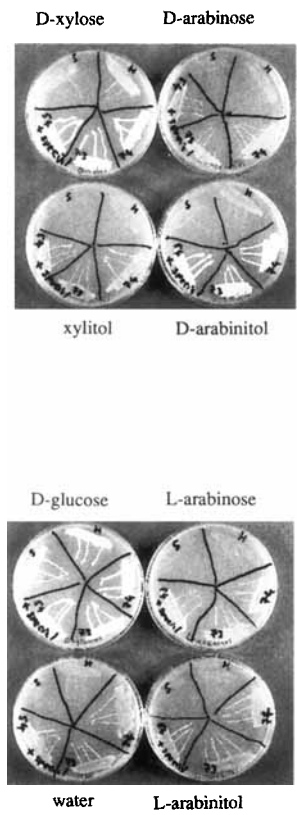

Figure 5. Growth of *P. stipitis* (CBS 6054, 5773 and 5774) and *S. cerevisiae* (S700 and H158) on plates containing either D-xylose, xylitol, D-arabinose, D-arabinitol, D-glucose (positive control), water (background), L-arabinose or L-arabinitol. The photograph was taken after 3 days of incubation at 30°C. Labels on plates: S=*S. cerevisiae* S700; H=*S. cerevisiae* H158; 54, 73 and 74=*P. stipitis*.

were used as references. After 3 days of incubation at 30°C, vigorous growth was only observed on plates containing either D-arabinitol, D-xylose or D-glucose. With *S. cerevisiae* S700 no growth was observed on plates containing D-arabinitol (growth only on D-glucose).

D-*Arabinitol dehydrogenase activity in* P. stipitis

P. stipitis has different *in vivo* polyol dehydrogenase activities depending on whether it grows on D-arabinitol, D-xylose or D-glucose (Table 1). ArDH activity is induced by D-arabinitol (0·78 U mg protein^{-1}) but not by D-xylose and D-glucose. Growth of *P. stipitis* on D-arabinitol also induced other polyol dehydrogenase activities such as xylitol and D-mannitol dehydrogenase activities (0·91 and 0·58 U mg protein^{-1}, respectively) (Table 1).

DISCUSSION

The zymogram staining method was used to screen a cDNA expression library directly on plaque replicas on nitro-cellulose membranes for polyol dehydrogenase activities. Using this method a D-arabinitol dehydrogenase (*ARDH*) gene was isolated. The λ-gt 11 cDNA expression library in *E. coli* was thus able to express the *ARDH* gene yielding an active enzyme even though the cDNA fragment contained a 5′-non-coding sequence with several stop codons before the ATG start site.

The *ARDH* gene was over-expressed in *S. cerevisiae* S700 yielding a specific activity in cell-free extract of 68·25 U per mg protein with D-arabinitol as substrate. The apparent $Km_{D\text{-arabinitol}}$ was half the value calculated for the *C. tropicalis* ArDH (20 mM and 40 mM, respectively), whereas the apparent Km_{NAD} was almost two-fold higher than the *C. tropicalis* ArDH (0·2 mM and 0·12 mM, respectively; Quong *et al.*, 1993). The substrate specificity of the two enzymes was similar with less than 10% cross-reactivities with other polyols, and the molecular weight of the two enzymes was also similar (30·0 kDa and 31·0 kDa, respectively). Thus, the two enzymes are probably also similar in structure. This is most likely also the case for the *C. albicans* ArDH with 85·5% identity to the *P. stipitis* ArDH. The ArDH from the bacterium *Klebsiella aerogenes* (Neuberger *et al.*, 1979), however, has a different polyol cross-reactivity pattern and a molecular weight of 46 000 Da.

The *S. cerevisiae* strain S700 expressing *ARDH* did not grow on plates containing D-arabinitol. *S. cerevisiae* may not be able to take up D-arabinitol from the medium or D-ribulose, the product of D-arabinitol oxidation by ArDH, may not be phosphorylated and thus not further metabolized. *P. stipitis* must have a specific D-ribulose kinase that *S. cerevisiae* may not have, since D-xylulokinase characterized from *P. stipitis* (Flanagan and Waites, 1992) did not cross-react with D-ribulose.

The proposed metabolic pathway of D-arabinitol in *P. stipitis* is shown in Figure 6. The first metabolic intermediate in D-arabinitol catabolism is D-ribulose, as shown in the present investigation. D-Ribulose must be phosphorylated by a rubulose kinase before entering the pentose phosphate pathway. Some fungi and yeasts produce D-arabinitol under special circumstances. With *P. stipitis* D-arabinitol production has been observed when the cells were grown on xylulose (Taylor

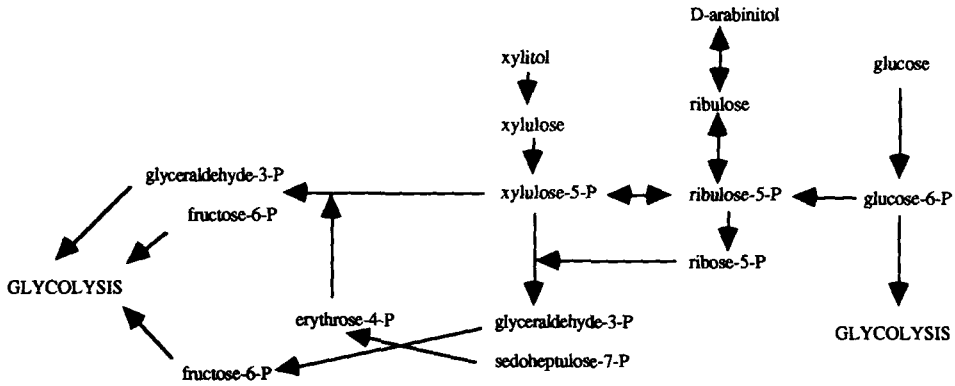

Figure 6. Proposed metabolic pathways of D-arabinitol metabolism of *P. stipitis* grown on either D-arabinitol, xylitol or D-glucose (respiratory inhibited).

et al., 1990) or when respiratory-inhibited *P. stipitis* was fermenting xylose (Jeppsson *et al.*, submitted). The anabolism of D-arabinitol with xylose-fermenting *P. stipitis* is not clear. Xylulose 5-phosphate, the third intermediate in xylose catabolism, can be epimerized to ribulose 5-phosphate by pentose phosphate epimerase, then dephosphorylated by a phosphorylase to ribulose and finally reduced to D-arabinitol by ArDH. Jovall *et al.* (1990) showed that D-arabinitol produced from radio-labelled glucose by the salt-tolerant yeast *Debaryomyces hansenii* was derived from ribulose-5-phosphate and that the major route was from decarboxylation of glucose-6-phosphate in the oxidative part of the PP (Figure 6).

The *in vivo* function of the *ARDH* gene in *P. stipitis* is not clear, but we suggest that the ArDH enzyme plays a role in xylose fermentation. Recombinant *S. cerevisiae* expressing *XYL1* and *XYL2* coding for xylose reductase and xylitol dehydrogenase, respectively, did not utilize xylose as efficiently as *P. stipitis* (Kötter and Ciriacy, 1993; Tantirungkij *et al.*, 1993; Meinander *et al.*, 1994). Recombinant *S. cerevisiae* over-expressing *TAL* (transaldolase) in addition to expressing *XYL1* and *XYL21* utilize xylose better, but still not as efficiently as *P. stipitis* (Walfridsson *et al.*, in preparation). In both cases xylitol formation was observed. The ArDH enzyme presently cloned and characterized can participate in the xylose metabolism by converting xylitol to xylulose by its xylitol dehydrogenase activity. In *C. tropicalis* high levels of D-arabinitol are produced during growth on *n*-alkane (Hattori and Suzuki, 1974). The oxidation of *n*-alkanes results in an accumulation of NAD(P)H creating a redox imbalance, which the cells respond to by producing D-arabinitol and thus regenerating $NAD(P)^+$. In *P. stipitis* CBS 6054 D-arabinitol was produced and excreted when the strain was fermenting xylose in the presence of a respiratory inhibitor (Jeppsson *et al.*, submitted), implying that D-arabinitol dehydrogenase could function as a redox balancer *in vivo* by reducing D-ribulose to D-arabinitol (Figure 6).

ACKNOWLEDGEMENTS

We would like to thank Prof. Hans Jörnvall (Karolinska Institutet, Stockholm) for valuable discussions. This work was supported by the Wallenberg Foundation, the Nordic Industrial Fund (86199), the National Swedish Board for Technical Development (90-00978), the Swedish National Science Research Council (EEG04777-300), and the Swedish Ethanol Foundation.

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