Characterization of the *Saccharomyces cerevisiae YMR318C* (*ADH6*) gene product as a broad specificity NADPH-dependent alcohol dehydrogenase: relevance in aldehyde reduction

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YMR318C represents an open reading frame from *Saccharomyces cerevisiae* with unknown function. It possesses a conserved sequence motif, the zinc-containing alcohol dehydrogenase (ADH) signature, specific to the medium-chain zinc-containing ADHs. In the present study, the *YMR318C* gene product has been purified to homogeneity from overexpressing yeast cells, and found to be a homodimeric ADH, composed of 40 kDa subunits and with a pI of 5.0–5.4. The enzyme was strictly specific for NADPH and was active with a wide variety of substrates, including aliphatic (linear and branched-chain) and aromatic primary alcohols and aldehydes. Aldehydes were processed with a 50-fold higher catalytic efficiency than that for the corresponding alcohols. The highest k_{cat}/K_m values were found with pentanal > veratraldehyde > hexanal > 3-methylbutanal > cinnamaldehyde. Taking into consideration the substrate specificity and sequence characteristics of the *YMR318C* gene product, we have proposed this gene to be called *ADH6*. The disruption of *ADH6* was not lethal for the yeast under laboratory conditions. Although *S. cerevisiae* is considered a non lignin-degrading organism, the catalytic activity of ADHVI can direct veratraldehyde and anisaldehyde, arising from the oxidation of lignocellulose by fungal lignin peroxidases, to the lignin biodegradation pathway. ADHVI is the only *S. cerevisiae* enzyme able to significantly reduce veratraldehyde *in vivo*, and its overexpression allowed yeast to grow under toxic concentrations of this aldehyde. The enzyme may also be involved in the synthesis of fusel alcohols. To our knowledge this is the first NADPH-dependent medium-chain ADH to be characterized in *S. cerevisiae*.

Key words: cinnamyl alcohol dehydrogenase, fusel alcohols, lignin metabolism, veratraldehyde.

INTRODUCTION

Alcohol dehydrogenases (ADHs) catalyse the reversible oxidation of alcohols to aldehydes or ketones with the corresponding reduction of NAD^+ or $NADP^+$. They are grouped into at least three families of enzymes; (1) medium-chain dehydrogenases/reductases (MDRs), containing approx. 350 residues per subunit; (2) short-chain dehydrogenases/reductases ('SDR'), with approx. 250 residues per subunit; and (3) iron-activated ADHs [1,2]. MDRs constitute a wide protein family with many different enzymic activities, including, among others, alcohol dehydrogenase, polyol dehydrogenase, glutathione-dependent formaldehyde dehydrogenase, cinnamyl alcohol dehydrogenase and quinone oxidoreductase activities [3]. Although some of the MDRs contain zinc and use this metal in the catalytic reaction, others, such as quinone oxidoreductases, do not.

The recent completion of several genome projects has allowed the search of putative MDRs to be carried out among the deduced open reading frames (ORFs). Thus the *Escherichia coli*, *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans* genomes have been screened for MDRs [4,5]. The analysis of the yeast genome for typical patterns of MDRs revealed 17 genes, which appeared to be members of this protein family [4].

MDRs that use zinc in their catalytic reaction possess a sequence motif known as the zinc-containing ADH signature [$GHEX_2GX_5(G,A)X_2(I,V,A,C,S)$; single amino acid residue notation, and where X is any amino acid residue] [6,7], including a histidine residue that is one of the ligands of the catalytic zinc. In a previous report [8], we used this motif to identify MDRs with zinc-dependent ADH activity in *S. cerevisiae*. The survey revealed 12 sequences with nine representing known MDR genes, including *ADH1* [9], *ADH2* [10–12], *ADH3* [13], *ADH5* [14], *SFA1* [15–17], *SOR1* [18], *YDL246C* (possessing 99% identity with *SOR1*), *XYL2* (a xylitol dehydrogenase) [19] and *BDH1* {a (2*R*, 3*R*)-2,3-butanediol dehydrogenase [8]}, and three putative ADHs with unknown function. The three MDR genes with unknown function were *YAL061W* (51% sequence identity with *BDH1*), *YMR318C* and *YCR105W* (64% identity between them). Among these 12 sequences, only *YMR318C* and *YCR105W* have the characteristics of an NADPH-dependent ADH. The five additional sequences found by Jörnvall et al. [4] are most probably MDRs that do not use zinc in their catalytic reaction.

In the present study, we report the characterization of a novel MDR gene, *YMR318C*, from the *S. cerevisiae* genome. In order to identify its function, the gene was overexpressed and the resulting enzyme was purified to homogeneity and its enzymic activity characterized. The enzyme was active towards a wide spectrum of linear, branched-chain and aromatic primary alcohols and aldehydes using NADPH as the coenzyme. These results demonstrate that *YMR318C* encodes an NADPH-dependent ADH. Further analysis, including the use of a null mutant strain, has been performed to determine its role in *S. cerevisiae* metabolism.

Abbreviations used: ADH, alcohol dehydrogenase; AspADH, *Acinetobacter* sp. strain M-1 ADH; Ateli3-2, *Arabidopsis thaliana* ELI3; bcADH, branched-chain ADH; CAD, cinnamyl ADH; DTT, dithiothreitol; EgCAD2, *Eucalyptus gunni* CAD2; IEF, isoelectric focusing; MDR, medium-chain dehydrogenase/reductase; MM, minimal medium; MbADH, *Myobacterium bovis* BCG ADH; ORF, open reading frame; Pceli, *Petroselinum crispum* ELI3; ScADHI *Saccharomyces cerevisiae* ADHI; ZmCAD2, *Zea mays* CAD2.

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EXPERIMENTAL

Materials

Restriction enzymes, T4-DNA ligase and alkaline phosphatase were from Roche Biochemicals (Mannheim, Germany) and Amersham Biosciences (Uppsala, Sweden). DNA Vent polymerase and *Hin*dIII linkers were from New England Biolabs (Beverly, MA, U.S.A.). The DNA oligonucleotides were synthesized and purified by Amersham Biosciences. Bacterial and yeast media were from Difco (Detroit, MI, U.S.A.) and US Biological (Swampsoncott, MA, U.S.A.). DEAE-Sepharose fast flow was from Sigma (St Louis, MO, U.S.A.); hydroxyapatite Biogel HT was from Bio-Rad (Hercules, CA, U.S.A.); Red Sepharose DL-6B and Superdex 200-HR were from Amersham Biosciences. $NADP^+$ (disodium salt) and NADPH (tetrasodium salt) were from Sigma. Alcohol and aldehydes substrates were obtained from Sigma, Aldrich (Saint Louis, MO, U.S.A.) or Fluka (Buchs, Switzerland), and were of the highest available quality.

Yeast and bacterial strains, and plasmids

For cloning procedures the *E.coli* XL1-Blue strain from Stratagene (La Jolla, CA, U.S.A.) was used. The *S. cerevisiae* yeast strain FY834α [20] was used to amplify the *YMR318C* gene (now designated *ADH6*; see the Results section) by PCR. The galactose-inducible *E. coli*-yeast shuttle vector pYes2 (Invitrogen, Groningen, The Netherlands), carrying the *URA3* selection marker and the promoter and upstream activating sequences of *GAL1*, was used to clone and overexpress the *YMR318C* gene in the protease-deficient yeast strain BJ2168 [21]. The pAAH5 vector, generously provided by Dr Benjamí Piña (Department de Biologia Molecular i Cellular, Centre de Investigació i Desenvolupament, Barcelona, Spain), was used to overexpress the *YMR 318C* gene product in medium containing glucose as the sole carbon source. This vector contains the *LEU2* selection marker and the *ADH1* promoter and terminator regions. The pCRscript vector (Stratagene, La Jolla, CA, U.S.A.) was used to make the construct to disrupt the *YMR318C* gene in yeast. *E. coli* was grown at 37 °C in Luria–Bertani broth supplemented with 50 μg/ml ampicillin to select for the desired plasmid constructs. Yeast cells were grown at 30 °C in minimal medium (MM) in the absence of leucine or uracil, further supplemented with 2% (w/v) glucose or galactose to allow for the selection and induction of the yeast transformed with the pAAH5 or pYes2 constructs respectively. Other media used for yeast growth were YPD [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose] and YPD with various concentrations of veratraldehyde.

Informatic programs

Amino acid sequence similarities were identified using the non-redundant version of the Swiss-Prot database, the translated version of the EMBL nucleotide sequence database ('TREMBL') and the FASTA search program [22]. The protein alignment was obtained using the GCG package [23].

Cloning methods

All DNA manipulations were performed under standard conditions as described previously [24,25].

Amplification of *YMR318C*

The *YMR318C* gene was amplified in a PCR reaction using genomic DNA from the strain FY834α as the template and the oligonucleotides, 5′-GGCGGATCCATCATGTCTTATCCTG-AGAAATTTGAAGG-3′ and 5′-GGGCTCGAGCTAGTAAA-ATTCTTTGTCG-3′, as primers. These primers were designed to introduce a *Bam*HI site at the 5′ end and a *Xho*I site in the 3′ end. The PCR reaction was started by heating the reaction mixture (1 μM each primer, 200 μM dNTPs and 3 mM $MgSO_4$) for 5 min at 95 °C prior to the addition of 10 units/ml of Vent DNA polymerase. This was followed by 30 cycles of 1 min at 95 °C, 1 min at 59 °C and 1 min of extension at 72 °C.

Construction of the pYes2-*YMR318C* and pAAH5-*YMR318C* vectors

The amplified PCR fragment, containing *YMR318C* flanked by *Bam*HI/*Xho*I sites, was purified from an agarose gel and digested with *Bam*HI and *Xho*I. This insert was subcloned into the pYes2 vector that had been digested with *Bam*HI and *Xho*I. The resulting construct was named pYes2-*YMR318C*. The construct was sequenced in both directions (Oswell DNA Services, Southampton, U.K.) to verify that there had been no mutations introduced by the PCR reaction.

To subclone *YMR318C* into the unique site of pAAH5, pYes2-*YMR318C* was digested with *Xho*I and, after making blunt-ends and ligating *Hin*dIII linkers, the vector was digested with *Hin*dIII and the resulting insert subcloned into *Hin*dIII-digested pAAH5. The construct was named pAAH5-*YMR318C*.

Construction of yeast strains overexpressing the *YMR318C* gene product

The yeast strain BJ2168 was grown in YPD media and transformed with the corresponding plasmids using the lithium acetate method [26]. Transformants were selected in MM supplemented with auxotrophic requirements [25] in the absence of uracil or leucine for pYes2 or pAAH5 plasmids respectively.

Purification of the *YMR318C* gene product

All purification steps were carried out at 4 °C. BJ2168 [pYes2-*YMR318C*] cells were inoculated into 5 litres of MM supplemented with 2% (w/v) galactose and auxotrophic requirements [25] with the exception uracil. Following incubation for 2 days, 23 g of cells were obtained and resuspended in 1 vol. of buffer A [20 mM Tris/HCl (pH 7) and 2 mM dithiothreitol (DTT)]. The crude extract was prepared with glass beads of 0.5 mm diameter on a bead-beater (Biospec Products, Bartlesville, OK, U.S.A.), with 4×1 min periods of disruption alternating with 3×1 min cooling periods. Glass beads were washed with 1 vol. of buffer A and the homogenate was centrifuged at 29000 ***g*** for 1 h at 4 °C. The supernatant was collected and dialysed against buffer A overnight at 4 °C. The dialysed extract was applied at 45 ml/h on to a DEAE-Sepharose column (2.4 cm × 21.5 cm) equilibrated with buffer A. The column was washed with 10 vol. of buffer A and the enzyme was eluted with a linear 0–0.3 M NaCl gradient in buffer A (600 ml). Fractions (5 ml) were collected and those with enzymic activity were pooled and concentrated to 12.5 ml using an Amicon concentrator (Danvers, MA, U.S.A.). The samples were directly loaded at 20 ml/h on to a hydroxyapatite column (1.5 cm × 13 cm) equilibrated with buffer B [10 mM potassium phosphate buffer (pH 7) and 0.5 mM DTT]. The column was washed with 200 ml of buffer B and the enzyme was eluted with a linear 10–400 mM KH_2PO_4 gradient in buffer B. The activity peak was collected and concentrated to 8 ml. The enzyme was loaded on to a PD-1 column (Amersham Pharmacia Biotech) equilibrated with a solution of 20 mM Hepes (pH 7.0) and 0.5 mM DTT (buffer C). The eluted protein was loaded at 35 ml/h on to a Red Sepharose column equilibrated with buffer C. The column was washed with 250 ml of buffer C and the enzyme was eluted with a linear 0–2.5 mM $NADP^+$ gradient in

buffer C. Fractions (5 ml) were collected and the purified Ymr318p fractions were pooled and concentrated. Finally, the cofactor was removed by applying the purified protein on to a Superdex 200-HR gel-filtration column (1 cm × 30 cm) connected to a Waters HPLC System, and the chromatography was performed in buffer D [50 mM sodium phosphate buffer (pH 7.0), 0.15 M NaCl, 0.5 mM DTT and 20 % (v/v) glycerol] at 0.4 ml/min. The pure protein was stored at −20 °C in buffer D. Protein concentration was determined by the Bradford method [27] using Bio-Rad protein assay reagents and BSA as the standard. The molecular mass of the purified enzyme was determined using the Superdex 200-HR gel-filtration column equilibrated with buffer D. Purified enzyme (45 μg) was loaded on to the column and the molecular-mass determined following calibration with gel-filtration molecular-mass markers (Sigma).

Enzyme activity

Enzyme activities were determined spectrophotometrically at 25 °C using a Cary 400 spectrophotometer (Varian, Sunnyvale, CA, U.S.A.). The activities towards different aldehydes were assayed in 0.6 ml reaction mixtures containing 33 mM sodium phosphate buffer (pH 7.0), 0.5 mM NADPH and 1 mM aldehyde (in 0.2 cm pathlength cuvettes). The decrease in A_{340} was followed to assess the enzymic activity towards most of the aldehydes, but at A_{365} when cinnamaldehyde or veratraldehyde were the substrates, and at A_{400} for coniferaldehyde and sinapaldehyde. The molar absorption coefficients (ϵ) used at pH 7.0 were: ϵ_{340} = 6.22 $mM^{-1} \cdot cm^{-1}$ and ϵ_{365} = 3.51 $mM^{-1} \cdot cm^{-1}$ for NADPH, ϵ_{365} = 7.71 $mM^{-1} \cdot cm^{-1}$ for vanillin plus NADPH, ϵ_{400} = 2.64 $mM^{-1} \cdot cm^{-1}$ for coniferaldehyde and ϵ_{400} = 2.00 $mM^{-1} \cdot cm^{-1}$ for sinapaldehyde. The activities with the alcohols were tested in 1 ml reaction mixtures containing either 0.1 M glycine (pH 10) or 33 mM sodium phosphate buffer (pH 7.0) with 1.2 mM NADP and 10 mM alcohol (in 1 cm pathlength cuvettes). Activities were measured at A_{340} (following the reduction of NADP) for most of the alcohols, but at A_{365} for the oxidation of cinnamyl alcohol and A_{400} for the oxidation of coniferyl alcohol (ϵ_{400} = 27.5 $mM^{-1} \cdot cm^{-1}$ at pH 10.0).

The influence of pH on the enzymic activities was carried out using either 33 mM sodium phosphate buffer (pH 6–7.5), 33 mM sodium pyrophosphate (pH 8–9) or 0.1 M glycine (pH 10–11). The substrates used were 1 mM cinnamaldehyde and 0.5 mM NADPH for the aldehyde reduction reaction, and 10 mM cinnamyl alcohol and 1.2 mM $NADP^+$ for the alcohol oxidation reaction. One unit of activity corresponded to 1 μmol of NADPH formed per min.

The steady-state parameters were determined by fitting the initial rates to the Michaelis–Menten equation using the non-linear regression program Grafit 3.0 (Erithacus Software Ltd., Horley, Surrey, U.K.). The kinetic constants are expressed as the mean ± S.E.M. of at least three separate experiments.

Disruption of the *YMR318C* gene

The inactivation of the *YMR318C* gene was carried out by one-step gene disruption [28] with the *TRP1* gene as a marker. The *YMR318C* gene was subcloned into the pCRscript vector (pCR-*YMR318C*) and the resulting construct was digested with two internal *Nde*I and *Nco*I restriction sites, releasing a 262 bp fragment. *TRP1* was obtained by digesting the YDpW plasmid [29] with *Bam*HI and, after making blunt-ends, it was inserted into the *Nde*I/*Nco*I sites of the digested pCR-*YMR318C* construct. The resulting plasmid was used as template to amplify by PCR the truncated *ymr318c* gene carrying the *TRP1* marker. The amplified linear fragment, containing *TRP1* flanked by homologous sequences to *YMR318C*, was used to transform the yeast strain BJ2168. The transformants were identified by growth on selective medium lacking tryptophan [25]. PCR analysis was carried out to confirm whether the replacement had occurred at the *YMR318* locus. The resulting *ymr318c::TRP1* strain was named BJ18.

Expression of Ymr318p in alternative carbon sources

Yeast strains BJ2168 and BJ18 (*ymr318c*Δ; designated *adh6*Δ in the Results section) were grown in 2 % (w/v) yeast extract, 2 % (w/v) peptone and either 2 % (w/v) galactose, 2 % (w/v) raffinose, 3 % (v/v) glycerol or 3 % (v/v) ethanol, and harvested in the exponential phase. After preparing the respective crude extracts, the enzymic activities towards the reduction of cinnamaldehyde in the presence of NADPH was measured and compared with the activities of the crude extracts of the same yeast strains grown in glucose. To ascertain whether the measured activities were due to the Ymr318p, equal amounts of protein from the crude extracts were analysed by native PAGE along with purified Ymr318p. The protein bands associated with NADPH-dependent reductase activity present in the gel were visualized using the method of Seymour and Lazarus [30]. The position of Ymr318p in the native gel was displayed by incubating the gel with 1 mM NADPH in 20 mM BisTris buffer (pH 7) and placing the gel in contact with a filter paper soaked in 10 mM pentanal. Gels were exposed to UV light and analysed for the disappearance of fluorescence, indicating the conversion of NADPH to NADP.

Elimination of veratraldehyde from yeast cultures

Cultures of BJ2168[pAAH5] (wild-type), BJ18[pAAH5] (null mutant) and BJ18[pAAH5-*YMR318*C] (overexpressing *YMR318C* in glucose medium) were grown in MM with glucose as the carbon and energy source and in the absence of leucine to select for the plasmids. When the D_{595} was 0.1, various concentrations of veratraldehyde (3, 5, 10 and 20 mM) were added. The growth curves were monitored by measuring the D_{595} and the degradation of veratraldehyde by following the disappearance of veratraldehyde at its maximum absorption wavelength (A_{310}), as described previously [31].

Electrophoretic analysis

SDS/PAGE was performed as described previously [32] using 12 % (w/v) acrylamide gels and the proteins were stained with silver nitrate. To ascertain the positions of the activity bands, proteins were separated by native PAGE on 7 % (w/v) acrylamide gels or by isoelectric focusing (IEF) [33] and the gels incubated in a solution containing 100 mM pentanol, 1.2 mM $NADP^+$, 0.1 mg/ml 5-methylphenazine methosulphate and 0.2 mg/ml Nitro Blue Tetrazolium (pH 8.6).

RESULTS

The *YMR318C* gene product is a member of the zinc-containing medium-chain ADH family

We have previously reported [8] that *YMR318C* from *S. cerevisiae* contains the $GHEX_2GX_5(G,A)X_2(I,V,A,C,S)$ sequence motif present in the zinc-containing medium-chain ADHs. A search performed with Ymr318p using the non-redundant Swiss-Prot database revealed over 50 % similarity to two recently characterized bacterial ADHs from *Myobacterium bovis* BCG (MbADH) [34] and *Acinetobacter* sp. strain M-1 (AspADH) [35]

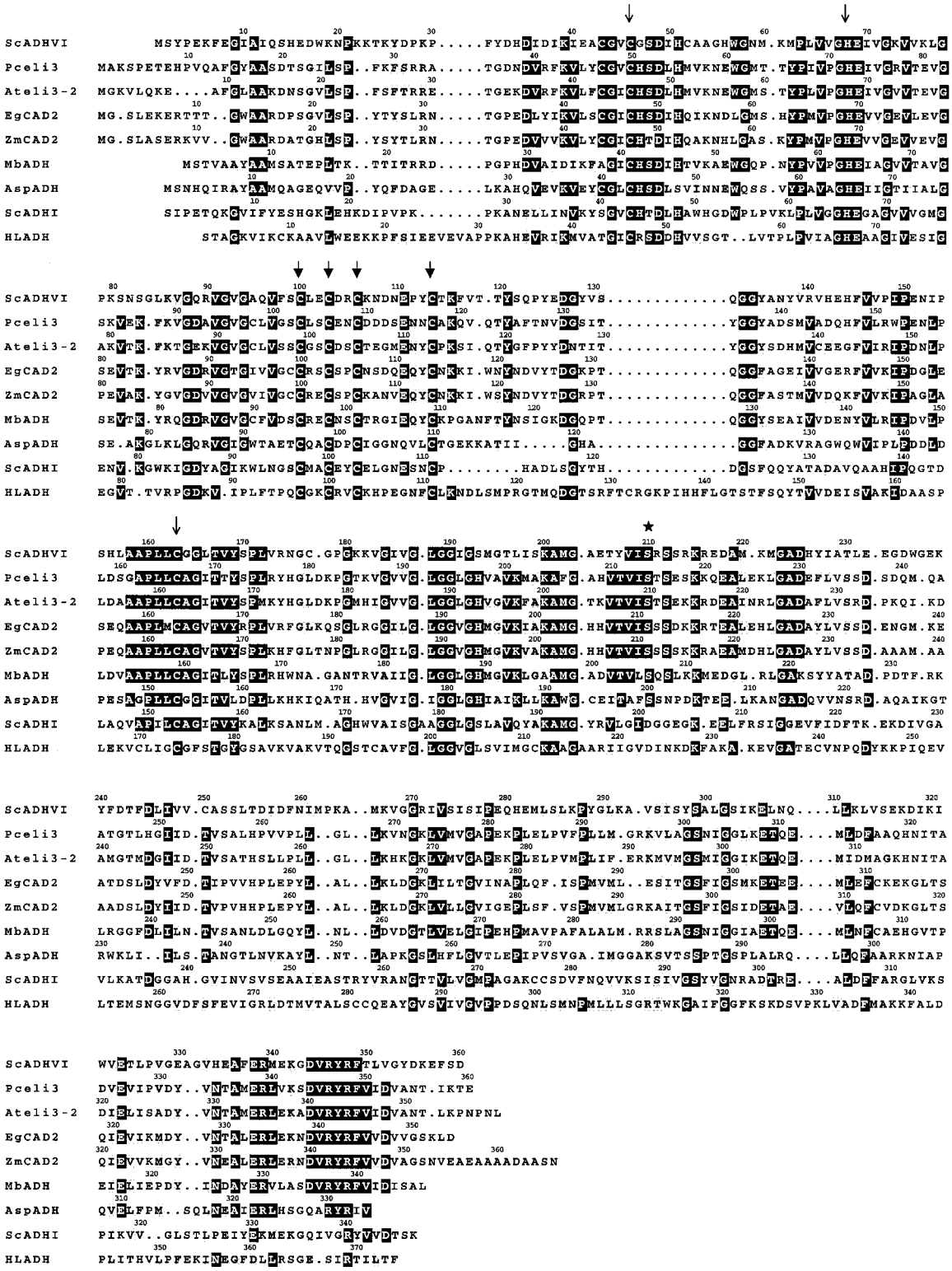

Figure 1 Alignment of *S. cerevisiae* ADHVI, encoded by the gene *YMR318C (ADH6)*, and related members of the MDR family

The alignment was obtained by using the PILEUP program. Residues in black boxes are identical, whereas those in grey boxes are similar, in at least six of the nine sequences aligned. The open arrows mark the amino acids involved in the binding of the 'catalytic zinc', whereas the solid arrows indicate the cysteine residues involved in the binding of the 'structural zinc'. Ser^{223} (using the numbering of horse liver ADH-EE) is characteristic of the NADPH dependent medium-chain ADHs. Protein sequences structurally related to the CADs are: ScADHVI, Pceli3, Ateli3-2, EgCAD2, ZmCAD2, MbADH and AspADH. The sequences ScADHI and horse liver ADH-EE (HLADH) are also shown. Pceli3 and Ateli3-2 are two enzymes implicated in defence reactions, whereas EgCAD2 and ZmCAD2 are two enzymes directly involved in the biosynthesis of lignin.

and with several plant cinnamyl ADHs (CADs); *Petroselinum crispum* ELI3 (Pceli) [36], *Arabidopsis thaliana* ELI3 (Ateli3-2) [37], *Eucalyptus gunni* CAD2 (EgCAD2) [38] and *Zea mays* CAD2 (ZmCAD2) [39]. A multiple sequence alignment between these proteins is shown in Figure 1, which also includes the *S. cerevisiae* ADHI (ScADHI) sequence. The horse liver ADH-EE enzyme (HLADH) was used as a standard for numbering. The *YMR318C* gene product had a characteristic feature of NADPH-dependent ADHs, namely a serine residue at position 223 instead of the aspartate or glutamate residues found in the coenzyme-binding site of the NADH-dependent ADHs (for example, ScADHI and ADH-EE). Ser^{223} is characteristic of some previously described $NADP^+$-dependent ADHs, such as Pceli3, Ateli3-2, EgCAD2 and ZmCAD2 (Figure 1) [36–39].

Overexpression of the *YMR318C* gene product

The pYes2-*YMR318C* plasmid, where the expression of *YMR 318C* was under the control of galactose, was employed to overexpress *YMR318C* in the protease-deficient yeast strain BJ2168. In order to confirm the expression and to follow the purification of the enzyme, a specific substrate was needed. Given the 37 % sequence identity existing between Ymr318p and different CADs, BJ2168 homogenates were assayed with some of the known substrates for CAD in the presence of NADPH. BJ2168 transformed with the pYes2 plasmid (BJ2168[pYes2]) served as a control. The crude extract from BJ2168[pYes2-*YMR318C*] had a 50-fold higher specific activity towards cinnamaldehyde than the BJ2168[pYes2] crude extract (3.9 units/mg versus 0.06 unit/mg). The crude extract from the *YMR318C*-overexpressing strain also had a 5–10 fold increase in specific activity towards various alcohols and aldehydes (see below) when compared with the non-overexpressing strain.

Overexpression of *YMR318C* was also confirmed by IEF followed by activity staining with 100 mM pentanol and $NADP^+$. With equal amounts of protein extracts loaded on to the gel, the overexpressing strain displayed an increased NADP-dependent pentanol dehydrogenase activity when compared with the non-overexpressing strain (results not shown). This increase in activity was associated with a protein band with a mobility identical to that of the purified protein in the IEF gel. Taking into account the substrate and cofactor specificity of the enzyme (see below), and its structural relationship with previously identified yeast ADHs, we designated the *YMR318C* gene as *ADH6* and its protein product as ADHVI.

Purification and properties of ADHVI

The cinnamaldehyde reduction reaction with NADPH as a cofactor was the chosen to follow the purification of ADHVI. The protein was purified to homogeneity by the following protocol; the supernatant of the crude extract was fractionated by separation on a DEAE-Sepharose column followed by hydroxyapatite and Red-Sepharose columns. NADP was used to elute ADHVI from the dye-affinity chromatography and was then itself removed by HPLC gel-filtration chromatography. This purification step led to an increase in the specific activity towards cinnamaldehyde, presumably due to the elimination of NADP that inhibited the NADPH-dependent reduction of cinnamaldehyde. Starting with 23 g of BJ2168[pYes2-*YMR 318C*] cells overexpressing *ADH6*, 1 mg of pure ADHVI was obtained (representing a 26 % yield), with a specific activity of the pure enzyme towards cinnamaldehyde of 183 units/mg (Table 1). The enzyme was stored at −20 °C in 20 % (v/v) glycerol and no loss of activity was detected over 1 month.

Table 1 Purification of *S. cerevisiae* ADHVI

ADHVI was purified from 23 g of yeast cells overexpressing *ADH6* and the activity after each purification step was measured in 33 mM sodium phosphate buffer (pH 7.0) containing 1 mM cinnamaldehyde and 0.5 mM NADPH. One unit of enzyme activity corresponds to 1 μmol of NADP formed per min.

	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Crude extract	183.0	717	3.9	1	100
DEAE Sepharose	34.5	702	20.3	5.2	98
Hydroxyapatite	7.1	231	32.4	8.3	32
Red Sepharose	1.2	123	106	27.1	17
Superdex 200-HR	1.0	183	183	47.0	26

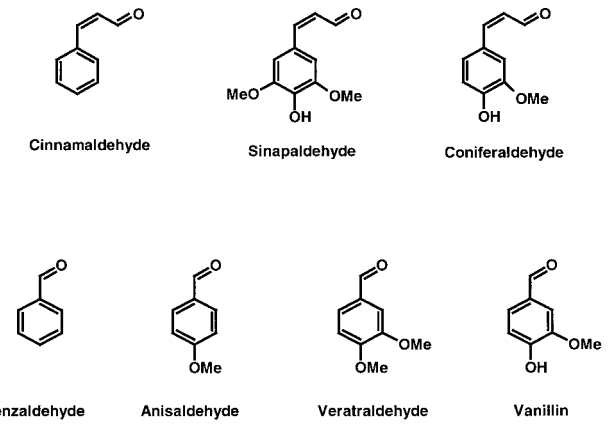

Figure 2 Chemical structures of several aldehyde substrates of *S. cerevisiae* ADHVI

Upper row: the structures of cinnamaldehyde and related compounds. Sinapaldehyde and coniferaldehyde, together with *p*-coumarylaldehyde (not shown), are the precursors of the corresponding cinnamyl alcohols that are the immediate precursors of lignin. Lower row: the structures of benzaldehyde and several derivatives. Anisaldehyde, veratraldehyde and vanillin are produced in the lignin biodegradation pathway.

Analysis by SDS/PAGE and subsequent silver nitrate staining showed a single band of approx. 40 kDa, which was close to the predicted molecular mass from the amino acid sequence (39613 Da). The native molecular mass was 71.3 kDa, as determined by gel-filtration chromatography. This result was also confirmed by native PAGE using gradient gels with 8–25 % (w/v) acrylamide. Thus the enzyme appeared to be a homodimer.

IEF experiments, followed by activity staining, revealed a major protein band of pI 5.4 and a minor band of pI 5.0 for the purified enzyme (results not shown).

ADHVI substrate specificity and kinetic parameters

The substrate specificity of the pure protein was tested with a broad range of aldehydes and alcohols (Figure 2 and Table 2). For the reduction reaction, the enzyme was active towards all of the aromatic and aliphatic (linear and branched-chain) aldehydes tested. Although the enzyme was very active towards aromatic aldehydes, such as cinnamaldehyde, benzaldehyde and substituted benzaldehydes, such as veratraldehyde and *p*-anisaldehyde, remarkably, it exhibited low activity towards substituted cinnamaldehydes, such as coniferaldehyde and sina-

Table 2 Substrate specificity of *S. cerevisiae* ADHVI

Reduction activities were measured in 33 mM sodium phosphate buffer (pH 7.0) containing 1 mM substrate and 0.5 mM NADPH. The activity towards cinnamaldehyde was taken as 100% with the specific activity being 183 units/mg of protein. Oxidation activities were measured in 0.1 M glycine at pH 10.0 containing 10 mM substrate (except for octanol where 1 mM was used) and 1.2 mM $NADP^+$. ND; not detected.

Reduction	Relative Activity	Oxidation	Relative Activity
Cinnamaldehyde	100	Cinnamyl alcohol	100
Sinapaldehyde	3	Coniferyl alcohol	27
Coniferaldehyde	10	Benzyl alcohol	45
Benzaldehyde	72	Ethanol	4
Veratraldehyde	82	Propanol	7
Anisaldehyde	72	Butanol	24
Vanillin	57	Pentanol	89
Propanal	21	Hexanol	154
Butanal	57	Octanol	52
Pentanal	109	2-Methylpropanol	1
Hexanal	89	2-(S)-Methylbutanol	7
Heptanal	88	3-Methylbutanol	15
Octanal	84	2-Butanol	2
2-Methylpropanal	67	2-Propanol	ND
2-Methylbutanal	92	1,2-Propanediol	ND
3-Methylbutanal	84	1,2-Butanediol	ND
trans-2-Nonenal	39	1,3-Butanediol	2
4-Hydroxynonenal	30	1,4-Butanediol	4
Furfural	27	2,3-Butanediol	ND
Methylglioxal	ND	Eugenol	ND
Acetone	ND		
Cyclohexanone	ND		

Table 3 Kinetic parameters of *S. cerevisiae* ADHVI

Enzymic activities were measured in 33 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM NADPH or 1.2 mM $NADP^+$ (for the reduction and oxidation reactions respectively). NADP and NADPH kinetics were performed with 10 mM cinnamyl alcohol and 1 mM cinnamaldehyde respectively. No saturation could be reached up to 0.5 M ethanol, 0.4 M pentanol or 0.19 M 3-methylbutanol at pH 10.

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1} \cdot min^{-1}$)
Cinnamaldehyde	0.172 ± 0.012	18400 ± 600	107200 ± 8600
Veratraldehyde	0.073 ± 0.003	15800 ± 140	217800 ± 9470
Hexanal	0.152 ± 0.017	21270 ± 920	139950 ± 16780
Pentanal	0.060 ± 0.009	22700 ± 190	380300 ± 57830
3-Methylbutanal	0.129 ± 0.035	14000 ± 670	109000 ± 30440
NADPH	0.029 ± 0.003		
Cinnamyl alcohol	0.436 ± 0.029	884 ± 90	2028 ± 247
Hexanol	9.1 ± 2.4	640 ± 92	71 ± 24
*Cinnamyl alcohol	0.700 ± 0.120	4200 ± 60	6000 ± 1000
*$NADP^+$	0.061 ± 0.002		

* Activities measured in 0.1M glycine (pH 10.0).

paldehyde. The enzyme had no activity with ketones, such as acetone or cyclohexanone. The specificity towards alcohols followed a similar pattern as for the aldehydes. Linear and branched-chain primary alcohols were substrates, whereas very low activity was found with secondary alcohols, such as butan-2-ol. The enzyme was inactive towards vicinal diols, such as 1,2-propanediol and 1,2-butanediol. For the aliphatic substrates, the activity increased with the length of the carbon chain until it reached five to six carbons. A further increase of the chain length produced a lower activity.

The maximum reduction activity with cinnamaldehyde was at pH 7.0, whereas the activity for the oxidation of cinnamyl alcohol increased steadily from pH 6–11 (results not shown). Kinetic parameters are given in Table 3. The highest catalytic efficiencies were observed for pentanal and veratraldehyde, although they were 2–3-fold lower for cinnamaldehyde, hexanal and 3-methylbutanal. When both reactions were monitored at pH 7.0, the oxidation of cinnamyl alcohol showed a catalytic efficiency 50-fold lower than for the reduction of the corresponding aldehyde. These values indicate that the major function of ADHVI is as an aldehyde reductase, rather than an alcohol dehydrogenase. The enzyme specifically required NADPH, which could not be substituted by NADH. Thus the activity displayed with NADH was $< 5\%$ of the activity with NADPH in both the reduction (with 0.5 mM cofactor) and oxidation (with 5 mM cofactor) reactions.

Construction of a null mutant *adh6*Δ strain

The disruption of *ADH6* with the *TRP1* marker [28] led to a null mutant *adh6*Δ strain. The isolation of genomic DNA from the *adh6*Δ strain, followed by PCR analysis, confirmed that the disruption had occurred at the *ADH6* locus. Specific amplification at the *ADH6* locus resulted in a gain of approx. 500 bp for the mutants due to the insertion of *TRP1*, which was bigger than the fragment removed. The disruption of *ADH6* was not lethal for *S. cerevisiae* when grown in YPD medium. Native PAGE showed that the null mutant *adh6*Δ strain did not display the activity band corresponding to ADHVI, whereas the wild-type strain did (results not shown).

Activity of ADHVI on alternative carbon sources

The specific activity of ADHVI from the yeast cells grown in raffinose was similar to that in yeast cells grown in glucose. The cells grown in galactose displayed a 3-fold increase in NADPH-dependent cinnamaldehyde reductase specific activity, which was due to the induction of ADHVI. When the cells were grown in glycerol, their NADPH-dependent cinnamaldehyde reductase specific activity increased approx. 4-fold with respect to the cells grown in glucose, although ADHVI expression was similar. The increase in activity was due to the induction of other enzymes with cinnamaldehyde reductase activity as visualized by PAGE analysis (results not shown). With ethanol as the carbon source, there was an approx. 15% decrease in the level of ADHVI activity, whereas at least one other enzyme with NADPH-dependent cinnamaldehyde reductase activity was induced (results not shown).

Yeast growth in medium containing veratraldehyde

The cell growth of the yeast strains BJ2168[pAAH5] (wild-type *ADH6*), BJ18[pAAH5] (isogenic to BJ2168, but containing *adh6*Δ) and BJ18[pAAH5-*YMR318C*] (overexpressing *ADH6* when grown in glucose) was followed in synthetic complete medium containing glucose [25] and various concentrations of veratraldehyde, a relevant compound in the ligninolysis pathway. Under these conditions, a specific activity of 7.9 units/mg of protein was obtained for the extract from BJ18[pAAH5-*YMR318C*], as opposed to 0.06 unit/mg obtained when *ADH6* was under the control of its own promoter in the wild-type yeast strain (BJ2168[pAAH5]). The specific activities of ADHVI from the wild-type cells (grown until mid-exponential phase) in the absence and presence of 5 mM veratraldehyde were similar (Figure 3). This result indicated that there was no induction of

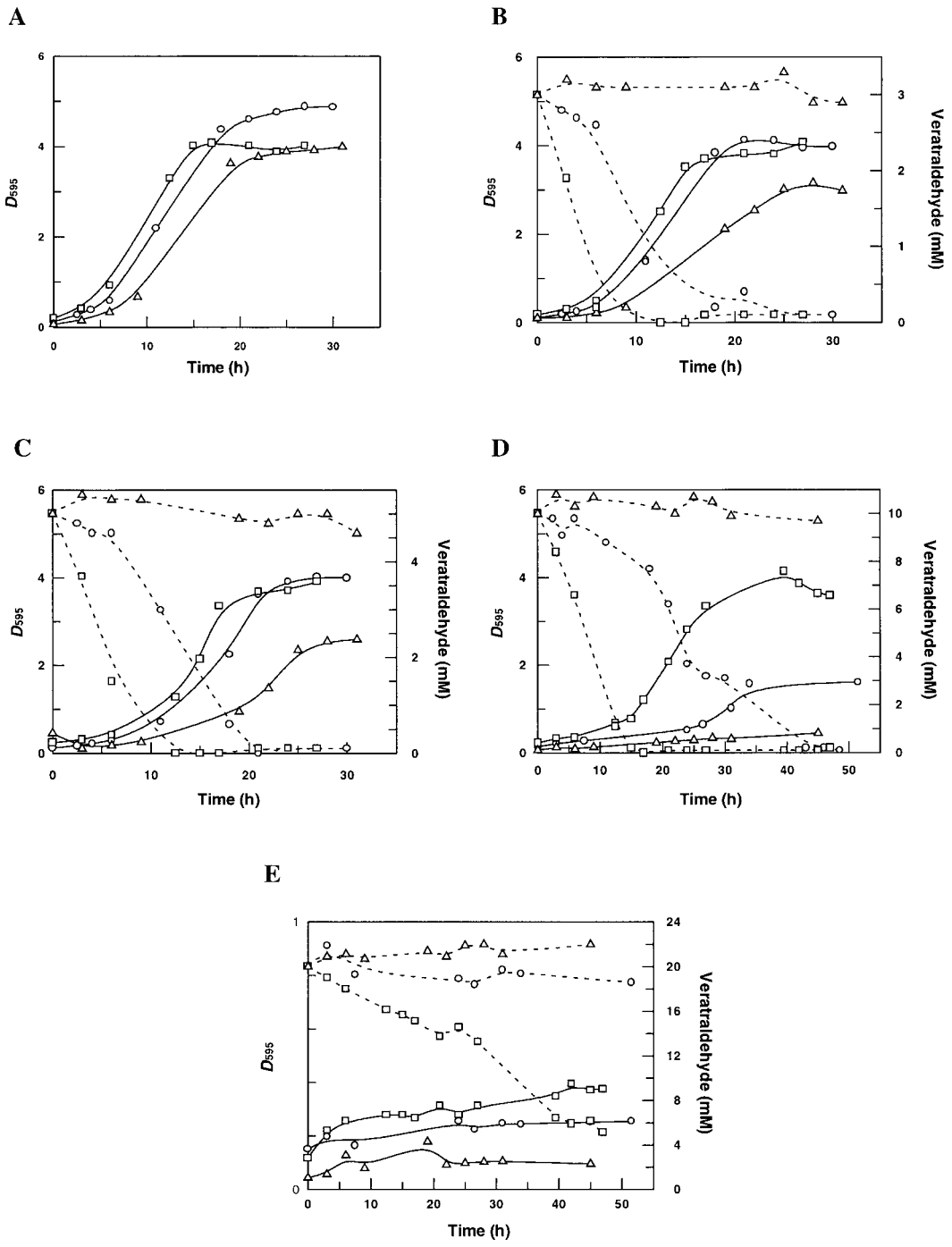

Figure 3 Veratraldehyde elimination and growth curves of different yeast strains at various concentrations of veratraldehyde

Cultures of BJ2168[pAAH5] (wild-type for *ADH6*; ○), BJ18[pAAH5] (the null mutant *adh6*Δ; △) and BJ18[pAAH5-*YMR318C*] (strain overexpressing *ADH6*; □) were grown in synthetic complete medium with 2% (w/v) glucose in the absence (**A**) or presence of 3 (**B**), 5 (**C**), 10 (**D**) or 20 mM (**E**) veratraldehyde. Growth curves (solid lines) and veratraldehyde concentrations (dashed lines) are shown for each culture.

ADHVI by veratraldehyde. The disappearance of veratraldehyde from the media was monitored spectrophotometrically. As shown in Figure 3, the *adh6*Δ strain (BJ18[pAAH5]) was unable to eliminate veratraldehyde at any concentration used over a 30 h time period. In contrast, the wild-type (BJ2168[pAAH5]) and *ADH6*-overexpressing strains (BJ18[pAAH5-*YMR318C*]) eliminated the aldehyde when it was present at 3, 5 and 10 mM in the culture medium (Figures 3B–D). At 20 mM veratraldehyde, however, only the strain overexpressing *ADH6* eliminated the aldehyde significantly (Figure 3E).

Veratraldehyde decreased the growth rate of the *adh6*Δ strain at all the concentrations used, suggesting a toxic effect of this aldehyde (Figure 3). At 3 mM and 5 mM veratraldehyde, the growth rates and the level of growth were reduced in the *adh6*Δ

strain but not in the wild-type and *ADH6*-overexpressing strains. The growth of these two strains with 3 mM and 5 mM veratraldehyde did not significantly differ from the controls without the aldehyde. At 10 mM veratraldehyde (Figure 3D), the reduction in growth rate and in the level of growth was also observed for the wild-type strain, but not for the *ADH6*-overexpressing strain. At 20 mM veratraldehyde, the inhibitory effect on yeast growth was observed for all strains. When a stationary culture of *adh6*Δ yeast cells, grown in 10 mM veratraldehyde, was observed by phase-contrast microscopy, a large number of enlarged and aggregated cells were observed. This was in contrast with the normal shape of most of the *adh6*Δ cells during the stationary phase without the aldehyde (results not shown).

It is apparent that there is a direct relationship between the growth rate and the veratraldehyde-elimination capacity of the yeast strains provided by ADHVI expression at the high concentration of aldehyde (Figure 3). These results also indicate that ADHVI is the only enzyme capable of significantly eliminating veratraldehyde in *S. cerevisiae in vivo*.

DISCUSSION

In a previous study [8], we had predicted, based on protein sequence comparisons, that the *YMR318C* gene product from *S. cerevisiae* would exhibit characteristics of the zinc-containing MDR family, and suggested it as a putative NADPH-dependent CAD. Recently, a phylogenetic tree constructed from all MDRs identified in *C. elegans*, *D. melanogaster*, *S. cerevisiae* and *E. coli*, placed the *YMR318C* gene product as an MDR related to CADs [5]. The multiple sequence alignment in Figure 1 shows that Ymr318p (now named ADHVI), exhibits characteristics of the zinc-containing MDR enzymes, such as conservation of Gly^{66}, Gly^{71}, Gly^{77} and Gly^{86} (after the horse liver ADH-EE numbering) in the substrate-binding domain, and Gly^{199}, Gly^{201}, Gly^{204} and Gly^{236} in the coenzyme-binding domain. It also has the three residues normally involved in the binding of the catalytic zinc and the four cysteine residues involved in the binding of the so-called 'structural zinc' (Figure 1). Although the presence of an aspartate or glutamate residue at position 223 is characteristic of NADH-dependent ADH [40–42], a serine residue at this position, as in ADHVI, has been assigned to NADPH-dependent ADHs [38]; a finding that has been confirmed from its kinetic properties in the present study.

To further classify ADHVI in the MDR family, we have aligned the sequence of the enzyme with the three most closely related types of proteins in the databases. All are MDRs and structurally related to CADs, including (1) Pceli3 [36] and Ateli3-2 [37], as representatives of the CADs implicated in defence mechanisms (both enzymes are expressed in response to elicitors in plant defence reactions); (2) EgCAD2 and ZmCAD2, as representatives of the CADs directly involved in the biosynthesis of lignin [43,44]; and (3) MbADH and AspADH, two bacterial ADHs structurally similar to CADs. While MbADH may have a role in the biosynthesis of the cell envelope lipids [34], AspADH appears to play a role in the metabolism of alkanes [35].

The homologous overexpression of the *ADH6* gene product, ADHVI, has allowed its purification to apparent homogeneity. Its appears to have a dimeric structure with the molecular mass of each subunit being 40 kDa. The purified ADHVI shows a strict specificity for NADPH and accepts a wide range of compounds as substrates, including linear and branched-chain primary alcohols and aldehydes, substituted cinnamyl alcohols and aldehydes as well as substituted benzaldehydes and their corresponding alcohols. The highest catalytic efficiencies, k_{cat}/K_m, were found for pentanal and veratraldehyde, whereas k_{cat}/K_m values for the oxidative reactions were 1–2 orders of magnitude lower. The specificity of the substrate and cofactor strongly supports the physiological involvement of ADHVI in aldehyde reduction rather than in alcohol oxidation.

The activity of ADHVI was monitored in the crude extracts of yeast cells grown in different carbon sources. The levels of ADHVI activity were similar for the cells grown in glucose, raffinose or glycerol, whereas they increased approx. 3-fold in cells grown in galactose. In contrast, a slight decrease in ADHVI activity was observed in the yeast cells grown in ethanol. These results are consistent with the levels of *YMR318C* gene transcripts found in cells grown in galactose, raffinose and ethanol (compared with glucose), as reported in a genomic expression study with DNA microarrays [45]. In a recent genome-wide transcriptional analysis performed with chemostat cultures of *S. cerevisiae*, it was observed that the level of the *YMR318C* gene transcript increased by approx. 15% under anaerobic conditions [46].

Two ADHs with similarities to ADHVI have been isolated previously isolated from *S. cerevisiae* [47,48]. The branched-chain ADH (bcADH) purified by Iersel et al. [48] has a pI of 5.9, a value that is close to the pI of the major band of ADHVI. Moreover, the bcADH was active in the reduction of linear, branched-chain and aromatic aldehydes; activities also shown by ADHVI. However, their coenzyme specificities and molecular masses are different. Thus their bcADH is a monomeric protein with a molecular mass of 37 kDa, whereas ADHVI is homodimeric with a molecular mass of 71.3 kDa. In addition, ADHVI showed strict specificity towards NADPH for the reduction reactions, whereas bcADH also utilizes NADH as a coenzyme. The ADH from *S. cerevisiae* isolated by Wales and Fewson [47] was NADP-dependent and showed activity towards some of the substrates of ADHVI. However, the fact that there is little overlap between the substrates studied by Wales and Fewson [47] and us (this report), and that the kinetic constants found for the common substrates were obtained with different experimental conditions, makes comparison difficult. Nevertheless, there seems to be a difference in the molecular masses, since the ADH isolated by Wales and Fewson [47] had an apparent monomeric structure with a molecular mass of 46.2 kDa.

The potential role of ADHVI in *S. cerevisiae* is not easy to ascertain when simply considering its structural similarity to CADs. Two different functions have been attributed to plant CADs; one group of CADs may be involved in the biosynthesis of the monomeric precursors of lignin from their cinnamyl aldehydes (*p*-coumaryl, coniferyl and sinapyl), whereas the other group may be implicated in plant-defence reactions. The activity profile of yeast ADHVI more closely resembles the activities shown by the defence-type CADs, namely a broad range substrate specificity, poor activity towards coniferaldehyde and sinapaldehyde and a 50–100-fold lower catalytic efficiency towards alcohols than for the corresponding aldehydes (Tables 2 and 3). However, both plant CAD types have been shown to be induced by wounding and by fungal infection [43], stimuli that also lead to lignification, whereas lignin has not been identified in yeast. Therefore it is unlikely that the role of yeast ADHVI is similar to that of plant CADs.

An alternative role for ADHVI might arise from its ability to convert veratraldehyde and anisaldehyde into their corresponding alcohols. In white rot fungi, such as *Phanerochaete chrysosporium*, the reduction of both aldehydes by aryl ADHs has been implicated in ligninolysis [49]. *S. cerevisiae* has seven ORFs with protein products showing high amino acid sequence similarity to aryl ADHs from *P. chrysosporium* [31]. However, a septuple-mutant strain constructed by the disruption of the seven ORFs

did not show a significant decrease in veratraldehyde degradation when compared with the wild-type [31]. The results shown in Figure 3 support the involvement of ADHVI in the *in vivo* dissimilation of veratraldehyde. Thus, whereas the null mutant *adh6*Δ strain did not modify the veratraldehyde concentration in the medium, the wild-type strain significantly decreased it, and the strain that overexpressed *ADH6* rapidly eliminated this aldehyde. Therefore ADHVI may give the yeast the opportunity to live in ligninolytic environments where products derived from lignin biodegradation may be available. The fact that other enzymic activities, such as the decarboxylase that converts ferulic acid and *p*-coumaric acid (two major lignin derivatives) into 4-hydroxy-3-methoxystyrene and vinylphenol respectively, have been found also in *S. cerevisiae* [31,50] would further allow this organism to use products derived from the biodegradation of lignin. In support of this hypothesis, a recent report [51] indicated a partial utilization of lignin as a carbon source by several yeast species, including *S. cerevisiae*.

Another potential function of ADHVI may be in the biosynthesis of fusel alcohols. Fusel alcohols are major flavour components in alcoholic beverages [52], and are derived either from the carbon backbones of several amino acids (valine, leucine, isoleucine and phenylalanine residues) or from α-ketoacids. They are produced by *S. cerevisiae* and other yeasts and include propan-1-ol, 2-methylpropan-1-ol, (S)-2-methylbutan-1-ol, 3-methylbutan-1-ol and 2-phenylethanol. Although there is no agreement in the literature on the yeast metabolic pathway that yields fusel alcohols [53,54], it appears clear that the last steps involve the reduction of propanal, 2-methylpropanal, (S)-2-methylbutanal and 3-methylbutanal to the corresponding fusel alcohols. Although other yeast ADHs, such as ADHI and ADHII, have been implicated in these reactions, our present results show that ADHVI could play a significant contribution to the pathway. In fact, the k_{cat}/K_m ratios of ADHVI towards these aldehydes are among the highest observed for all of the substrates tested.

In summary, we have identified in the yeast genome a new member of the growing MDR family and have demonstrated that the corresponding protein is an NADPH-dependent ADH of broad substrate specificity. Given the higher k_{cat}/K_m ratios for aldehyde reduction (in comparison with alcohol oxidation) found for ADHVI, together with the high [NADPH]/[NADP] ratio necessary for the growth of *S. cerevisiae* [55], its physiological function is most probably the reduction of aromatic and medium-chain aliphatic aldehydes. Our results support the involvement of this enzyme in the biosynthesis of fusel alcohols and in the final steps of the lignin degradation pathway. To our knowledge, ADHVI is the first NADPH-dependent member of the MDR family identified in yeast.

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