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Molecular Cloning, Characterization, and Potential Roles of Cytosolic and Mitochondrial Aldehyde Dehydrogenases in Ethanol Metabolism in *Saccharomyces cerevisiae*†

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The full-length DNAs for two *Saccharomyces cerevisiae* aldehyde dehydrogenase (ALDH) genes were cloned and expressed in *Escherichia coli*. A 2,744-bp DNA fragment contained an open reading frame encoding cytosolic ALDH1, with 500 amino acids, which was located on chromosome XVI. A 2,661-bp DNA fragment contained an open reading frame encoding mitochondrial ALDH5, with 519 amino acids, of which the N-terminal 23 amino acids were identified as the putative leader sequence. The ALDH5 gene was located on chromosome V. The commercial ALDH (designated ALDH2) was partially sequenced and appears to be a mitochondrial enzyme encoded by a gene located on chromosome XV. The recombinant ALDH1 enzyme was found to be essentially NADP dependent, while the ALDH5 enzyme could utilize either NADP or NAD as a cofactor. The activity of ALDH1 was stimulated two- to fourfold by divalent cations but was unaffected by K^+ ions. In contrast, the activity of ALDH5 increased in the presence of K^+ ions: 15-fold with NADP and 40-fold with NAD, respectively. Activity staining of isoelectric focusing gels showed that cytosolic ALDH1 contributed 30 to 70% of the overall activity, depending on the cofactor used, while mitochondrial ALDH2 contributed the rest. Neither ALDH5 nor the other ALDH-like proteins identified from the genomic sequence contributed to the in vitro oxidation of acetaldehyde. To evaluate the physiological roles of these three ALDH isoenzymes, the genes encoding cytosolic ALDH1 and mitochondrial ALDH2 and ALDH5 were disrupted in the genome of strain TWY397 separately or simultaneously. The growth of single-disruption Δ*ald1* and Δ*ald2* strains on ethanol was marginally slower than that of the parent strain. The Δ*ald1* Δ*ald2* double-disruption strain failed to grow on glucose alone, but growth was restored by the addition of acetate, indicating that both ALDHs might catalyze the oxidation of acetaldehyde produced during fermentation. The double-disruption strain grew very slowly on ethanol. The role of mitochondrial ALDH5 in acetaldehyde metabolism has not been defined but appears to be unimportant.

Yeast aldehyde dehydrogenase (ALDH) has been studied for many years. In the 1950s it was found that a K^+ ion-activated enzyme existed, and many kinetic properties of the enzyme were studied by different investigators (2, 3, 21). Most of the interest was focused on understanding the metabolism of acetaldehyde derived from the oxidation of ethanol during the aerobic growth of the organism. More recently, a different form of ALDH was identified which primarily oxidized long-chain aldehydes and was not involved in acetaldehyde oxidation (1, 25).

Our laboratory reported on the cloning and sequencing of a yeast ALDH (19) and suggested it was mitochondrial in origin. When we started to reinvestigate the project, we came to realize that the DNA we sequenced could not have been totally correct. Hence, we rescreened our libraries and found two new DNAs which would code for different enzyme forms. The complete *Saccharomyces cerevisiae* genome has recently been published (7), and it verified that our original clone was not correct but that the two new genes we had found were present. Here we report data on the cloning, expression, purification, and characterization of these two enzyme forms. Neither is the commercially available K^+-activated enzyme.

During growth on ethanol it is necessary for *S. cerevisiae* to convert two carbons from ethanol to acetate so they can be used by the glyoxylate system to incorporate the carbons into usable metabolites. The total genomic sequence of *S. cerevisiae* S288C revealed that seven genes could code for proteins which appear to be members of the ALDH family. The recombinantly expressed enzymes encoded by the two genes we cloned appeared to have properties similar to enzymes found by other investigators. One enzyme was shown to be localized to the mitochondria, with properties somewhat similar, but not identical, to those reported by Black in 1951 (2). This enzyme was also shown to exhibit properties different from those of the commercially available enzyme. The second enzyme was localized to the cytosol, and had properties very similar to those of the enzyme described by Seegmiller (20) and Dickinson (4). The data also suggest that the acetaldehyde produced during ethanol oxidation could be converted into acetate in the cytosol as well as in the mitochondria. The contribution of the enzymes may be influenced by the NADP/NAD ratio in the cells.

Investigators have suggested that mitochondrial ALDH was involved in the oxidative metabolism of ethanol while cytosolic ALDH was responsible for the oxidation of acetaldehyde produced during fermentation (15, 18), although the bulk of acetaldehyde is reduced to ethanol. We have used the nomenclature previously adopted (31) to identify the yeast enzymes described in this study based on their amino acid sequence homologies with and enzymatic similarities to the mammalian enzymes. We have disrupted the cytosolic ALDH gene (des-

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype
XK25-1B	*MAT*α *ura3-52*
S288C	*MAT*α *SUC2 mal1 gal2 CUP1*
Parent TWY397	*MAT*α *ura3 his7 trp1 leu2*
Single disruption	
HWT6	*MAT*α *ura3 his7 trp1 leu2* Δ*ald1*::*TRP1*
HWL18	*MAT*α *ura3 his7 trp1 leu2* Δ*ald5*::*LEU2*
HWH11	*MAT*α *ura3 his7 trp1 leu2* Δ*ald2*::*HIS7*
Double disruption	
HWTH2	*MAT*α *ura3 his7 trp1 leu2* Δ*ald1*::*TRP1* Δ*ald2*::*HIS7*
HWHL1	*MAT*α *ura3 his7 trp1 leu2* Δ*ald2*::*HIS7* Δ*ald5*::*LEU2*
HWTL1	*MAT*α *ura3 his7 trp1 leu2* Δ*ald1*::*TRP1* Δ*ald5*::*LEU2*
Triple disruption HWTHL10	*MAT*α *ura3 his7 trp1 leu2* Δ*ald1*::*TRP1* Δ*ald2*::*HIS7* Δ*ald5*::*LEU2*

ignated *ALD1*) and two mitochondrial ALDH genes (designated *ALD2* and *ALD5*) in the *S. cerevisiae* genome and found that ALDH1 and ALDH2 were indeed important for the metabolism of ethanol. Both ALDHs might be involved in the oxidation of acetaldehyde formed during fermentation. The physiological role of ALDH5 in acetaldehyde metabolism was not completely elucidated in this study.

MATERIALS AND METHODS

Materials. NAD, NADP, uracil, tryptophan, leucine, and histidine were purchased from Sigma Chemical Co.; isopropyl-β-D-thiogalactoside (IPTG) was purchased from Fisher Scientific; propionaldehyde and acetaldehyde were purchased from Aldrich Chemical Co., Inc.; isoelectric focusing (IEF) standards and prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) standards were purchased from Bio-Rad Laboratories, Inc.; agarose IEF gel and Pharmalytes were purchased from Pharmacia Biotech Inc.; a Sequenase version 2.0 kit was purchased from United States Biochemical Corp.; commercial K^+ ion-activated ALDH was purchased from Boehringer Mannheim; Freund's adjuvant was purchased from Behring Diagnostics; restriction enzymes were purchased from Promega Corp. or New England Biolabs; a Frozen-EZ yeast transformation kit was purchased from Zymo Research. The GenBank accession numbers for *ALD1*, *ALD2*, and *ALD5* are U56604, Z75282, and U56605, respectively.

S. cerevisiae **strains and growth conditions.** *S. cerevisiae* XK25-1B (*MAT*α *ura3-52*) and S288C were kindly provided by Gunter Kohlhaw of the Department of Biochemistry at Purdue University. *S. cerevisiae* TWY397 (33) was obtained from Ted A. Weinert's laboratory at the University of Arizona. The yeast strains used and constructed in this study are listed in Table 1. Overnight cultures were grown in YEPD (1% yeast extract, 2% peptone, and 2% glucose) medium. The cultures were serially diluted the next day with SD medium (0.67% yeast nitrogen base without amino acids and 2% glucose), and a volume containing approximately 100 colonies was spread on SD plates. Colony sizes were estimated from the measurement of five separate colonies after growth for the indicated time periods. In some experiments 2% glucose in SD medium was replaced with 2% ethanol, 2% acetate, or 2% glycerol, as indicated. Uracil (20 μg/ml), tryptophan (40 μg/ml), leucine (60 μg/ml), and histidine (20 μg/ml) were added to the media to support the growth of the strains when needed. *Escherichia coli* JM109(DE3) and BL21(DE3) were used for expression of *S. cerevisiae* cytosolic ALDH1 and mitochondrial ALDH5, respectively.

PCR to amplify a fragment encoding ALDH1. *S. cerevisiae* chromosomal DNA was prepared according to the method of Hoffman (14). PCR was performed with a DNA thermal cycler (Perkin-Elmer) for 30 cycles at 94°C for 30 s, 46°C for 30 s, and 74°C for 1 min. *S. cerevisiae* chromosomal DNA served as the template, and the following two degenerate oligonucleotides served as primers: 5′ primer, TTTGAACATATGGCT(C or A)TTCACA(C)GGT(C)TCC(T or G)ACT, containing a *Nde*I site (underlined), and 3′ primer, TTTGGATCCA(C)ACTGGA(C or T)CCGAAA(G)ATT(C)TCT(C)TC, containing a *Bam*HI site (underlined).

The 0.5-kb fragments were digested with *Nde*I and *Bam*HI and subcloned into the pT7-7 vector (34). The fragments were sequenced with the Sequenase version 2.0 kit and was labeled with [α-^{32}P]dCTP by the Prime-a-Gene labeling system, following the instructions from Promega.

S. cerevisiae **library screening.** Approximately 3 × 10^6 cells from the *S. cerevisiae* YEP 24 genomic library, RB366, which was constructed from *S. cerevisiae* DBY939 (*suc2-215am*), were screened by using [α-^{32}P]dCTP-labeled probe, following the methods described previously (8–10). Plasmids isolated from positive colonies were digested with a series of restriction enzymes and cloned into a vector for DNA sequencing in both directions, using the Sequenase version 2.0 kit.

Cloning the full-length DNAs encoding *S. cerevisiae* cytosolic and mitochondrial ALDHs into expression vectors. PCR was used to generate the coding regions of ALDHs from the vectors carrying the genes for the *S. cerevisiae* cytosolic and mitochondrial ALDHs, respectively. The two primers for cytosolic ALDH1 were 5′TTTGAATTCCATATGACTAAGCTACACTTTGAC3′ (5′ primer containing an *Nde*I site [underlined]) and 5′TTTGGATCCTTACAACTTAATTCTGACAGC3′ (3′ primer containing a *Bam*HI site [underlined]). The 1.5-kb PCR product was digested with *Nde*I and *Bam*HI and was cloned into the *Nde*I/*Bam*HI sites of the pT7-7 expression system (designated pT7-7-yALDH1) as described previously (34). The full-length DNA for ALDH was sequenced to verify the absence of any misincorporations produced by the PCR.

To construct the expression vector for the *S. cerevisiae* mitochondrial ALDH5 lacking 23 amino acids of the leader sequence, a 1.8-kb fragment was synthesized by PCR with 5′-CCAGATCCATATGTCTCAAGCACCATTACGCGTTCC (5′ primer containing an *Nde*I site [underlined]) and 5′-CCATCGATTCAACGAATTGGCTTGTCAATG (3′ primer containing a *Cla*I site [underlined]). The purified 1.8-kb *Nde*I-*Cla*I fragment was then inserted into the plasmid pT7-7 previously digested with *Nde*I and *Cla*I. The resulting plasmid (designated pT7-7-yALDH5) was transformed into *E. coli* BL21(DE3).

Cloning of *S. cerevisiae HIS7* gene. The coding sequence of *HIS7* was cloned by PCR and sequenced by following standard procedures.

Cloning of *S. cerevisiae* gene encoding ALDH2. Based on the *ALD2* sequence published in GenBank, two primers were designed to amplify the coding region of *ALD2* plus 387-bp upstream and 155-bp downstream sequences. The 5′ primer, 5′-CCGGAATTCACCGTTTTGTGTAACA, contained an *Eco*RI site (underlined), and the 3′ primer, 5′-CGCGGATCCGAAAATTGATTATCGGGAG, contained a *Bam*HI site (underlined). PCR was performed with *S. cerevisiae* chromosomal DNA as the template. A 2.1-kb PCR product was digested with *Eco*RI and *Bam*HI and then cloned into the *Eco*RI/*Bam*HI site of plasmid pSP72 (Promega). The new plasmid was called WA1. About 200 bp from the each end of the PCR product was sequenced to verify that we had cloned the *ALD2* gene. No misincorporations were detected.

Confirmation of the ALDH5 gene DNA sequence. Alan B. Rose's laboratory (Princeton University) kindly provided us with a plasmid containing a gene encoding a possible ALDH (18a). To prove whether this was really an unknown *S. cerevisiae* ALDH gene, a 2.6-kb fragment that contained the putative *ALD* gene was subcloned onto a Bluescript II SK plasmid and sequenced from both directions. A 1.44-kb *Sma*I-*Bam*HI fragment was synthesized by PCR amplification with 5′-GGTGACAAGATAGGTACCCGGGTCAGTGCA (including a *Sma*I site [underlined]) and 5′-GCACAAAACAATGACTTACCAGGATCCATTGACTCAATG (including a *Bam*HI site [underlined]) as the two primers. This fragment included 1.05 kb of the 5′ upstream region and 129 amino acids of the *ALD5* coding region.

Disruption of *ALD1*, *ALD2*, and *ALD5* genes from *S. cerevisiae* chromosomes. A 1.2-kb fragment of the coding sequence for ALDH1 was removed from the expression plasmid and replaced by a 1.2-kb fragment containing the selectable *TRP1* gene. The fragment used for the subsequent transformation and disruption of the yeast *ALD1* gene contained 0.4 kb of 5′ and 0.8 kb of 3′ homologous sequences for integration. A 0.55-kb fragment of the coding sequence for ALDH2 was removed from the expression plasmid and replaced by a 2.0-kb fragment containing the selectable *HIS7* gene. The fragment used for the subsequent transformation and disruption of the yeast *ALD2* gene contained 1.0 kb of 5′ and 0.6 kb of 3′ homologous sequences for integration. A 1.2-kb fragment of the coding sequence for ALDH5 was removed from the expression plasmid and replaced by a 2.3-kb fragment containing the selectable *LEU2* gene. The fragment used for the subsequent transformation and disruption of the yeast *ALD5* gene contained 0.6 kb of 5′ and 0.4 kb of 3′ homologous sequences for integration.

Expression of DNA encoding *S. cerevisiae* cytosolic or mitochondrial ALDHs. Vectors which carried the DNAs encoding *S. cerevisiae* cytosolic or mitochondrial ALDHs were expressed in JM109(DE3) and BL21(DE3), respectively. YT medium (2×) (19a) containing 50 μg of ampicillin/ml was inoculated with an overnight culture. The cells were grown for about 5 h at 37°C, and then IPTG was added to a final concentration of 0.4 mM. Growth was continued for about 16 h at 16°C after the addition of IPTG.

Purification of recombinantly expressed *S. cerevisiae* ALDHs. The crude lysates of *E. coli* cells harboring the expression vectors for *S. cerevisiae* cytosolic and mitochondrial ALDHs were first treated with 5% protamine sulfate. After dialysis, samples were chromatographed on DEAE-cellulose equilibrated with 10 mM sodium phosphate, pH 7.4, containing 10% glycerol, 1 mM EDTA, and 0.025% β-mercaptoethanol. After the column was washed with equilibration buffer, ALDH was eluted with a NaCl gradient (0 to 200 mM NaCl in equilibration buffer). The fractions containing ALDH (as measured by enzyme activity) were collected; dialyzed against a solution containing 50 mM sodium phosphate (pH 7.0), 10% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol, and 50 mM

NaCl; and then applied to Blue Sepharose CL-6B. The column was eluted with a sodium chloride gradient (0.05 to 1.05 M). Fractions containing the enzyme were pooled and dialyzed against 100 mM sodium phosphate (pH 7.4)–0.1 mM dithiothreitol to remove NaCl and stored in 50% glycerol at −20°C. The concentration of protein was determined by the Bio-Rad protein assay with bovine serum albumin as a standard.

Preparation and isolation of antibodies against *S. cerevisiae* cytosolic and mitochondrial ALDHs. ALDHs partially purified through DEAE-cellulose were subjected to SDS-PAGE. The ALDH bands were excised, sliced, and lyophilized following the methods described previously (11). After mixing with Freund's adjuvant, two 2-mg/ml samples were used to immunize two rabbits through subcutaneous injections. After the immunization was boosted twice, serum was collected. Antibody was purified by ammonium sulfate precipitation and batch separation on DEAE-cellulose (12).

Dehydrogenase activity assay. The dehydrogenase activity assay was performed with an Aminco filter fluorometer and 100 mM sodium phosphate buffer, pH 7.4 (30). The activity was recorded as the formation of NADPH or NADH as a function of time. Metal ion effects on the dehydrogenase activity of cytosolic ALDH1 were determined in 100 mM Tris buffer, pH 7.4, with different concentrations of NaCl, KCl, $MnCl_2$, MgCl, $ZnCl_2$, and $CaCl_2$, while K^+ ion effects on the mitochondrial ALDH5 were determined in 100 mM sodium phosphate buffer, pH 7.4.

IEF slab gel. IEF was performed on a Pharmacia flatbed electrophoresis apparatus at 10°C (5), using Pharmalyte, pH 4.5 to 5.4. After electrophoresis was completed, the IEF agarose gel was stained for activity with 100 mM sodium phosphate (pH 7.4)–20 mM KCl containing 5 mM NADP and 5 mM propionaldehyde. The IEF standards were stained with Coomassie blue.

Western blot analysis. The crude lysates of strains were applied to SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes and probed with antibodies prepared against ALDH1 or ALDH5.

MALDI mass spectroscopy. The matrix for mass spectroscopy was made up as a solution of 10 mg of sinapinic acid/ml in 50% acetonitrile–0.1% trifluoroacetic acid. Purified ALDH1 and ALDH5 in phosphate buffer were dialyzed against double-deionized water. Protein samples (1 μl) at a concentration of about 1 pmol/μl were mixed with 1 μl of matrix on the sample well and allowed to dry. MALDI mass spectra were obtained on a Voyager biospectrometry workstation (Perseptive Biosystems, Framingham, Mass.). The experiments were performed at the Mass Spectrometry Laboratory in the Department of Biochemistry, Purdue University.

RESULTS

Cloning studies. (i) PCR to find the DNA sequence encoding a *S. cerevisiae* ALDH. It has been reported that *S. cerevisiae* contains both mitochondrial and cytosolic ALDH, identified by the ALDH activity assay after cell fractionation (15). In an attempt to clone cytosolic ALDH, PCR was performed with *S. cerevisiae* chromosomal DNA as the template. Based on the highly conserved regions found in all ALDHs sequenced so far (13), two degenerated primers were designed to amplify a 0.5-kb fragment. Sequence homology analysis revealed that the fragment was 60% identical to rat and human mitochondrial ALDH2 and cytosolic ALDH1. Thus, it appears that we identified the DNA encoding a *S. cerevisiae* ALDH which corresponded to a gene on chromosome XVI (Genbank accession no. U56604).

(ii) Nucleotide sequence of *S. cerevisiae ALD1* gene. A *S. cerevisiae* genomic library was screened by using the fragment labeled with [α-^{32}P]dCTP. Positive colonies were identified after the second screening. The plasmids isolated from positive colonies were digested with different restriction enzymes to determine the size of the insertion which was subcloned into the plasmid pYEP24. Finally, the insertion (about 7.8 kb) was digested with *Nde*I and *Xba*I and fragments from the digestion were cloned into the pT7-7 vector for protein expression. The three fragments from the *Nde*I digestion were sequenced, and the middle 2.9-kb fragment (called pS20) was found to contain the *S. cerevisiae* cytosolic *ALD1* gene. DNA sequencing was performed from both directions, and a 2,744-bp sequence was found to contain the full-length sequence of a *S. cerevisiae* enzyme, which we called ALDH1 (Fig. 1).

The open reading frame was predicted to encode 501 amino acids, including the initiation methionine, with a calculated molecular mass of 54,582.6 Da. Inspection of the sequence showed that the protein did not contain a putative leader sequence; hence, we assumed the enzyme was of cytosolic origin.

ALD1

amino acid position

121-122 TTTAAG (This study)
F K

TTA (GenBank)
L

ALD5

48 ACT (This study)
T

ATC (GenBank)
I

90-103

CTAAAAAAGCTTTTGAAACGAAGTGTCTATTGTAGAGCCGGA (This study)
L K K L L K R S V Y C R A G
GCTAAAAAAGCTTTTGAAACGAAGTGGTCTATTGTAGAGCCGGAG (GenBank)
A K K A F E T K W S I V E P E

410 GAA (This study)
E

GGA (GenBank)
G

FIG. 1. Differences between nucleotide and deduced amino acid sequences for *ALD1* and *ALD5* from this study and GenBank. Deduced amino acids for each nucleotide sequence are shown below their respective codons.

(iii) Nucleotide sequence of the *S. cerevisiae ALD5* gene. The plasmid sent by A. B. Rose's laboratory was sequenced and found to contain a full-length DNA encoding an ALDH gene we designated *ALD5*. The sequence of *ALD5* was almost identical (Fig. 1) to a *S. cerevisiae* sequence published in GenBank (accession no. U56605), which was located on chromosome V. Analysis of this sequence revealed a longer open reading frame of 1,560-bp predicted to encode a polypeptide of 519 amino acids with a calculated molecular mass of 56,540 Da. Inspection of the sequence showed that the protein contained a putative mitochondrial leader sequence. This was confirmed by fusion of the putative leader sequence to β-galactosidase and its subsequent import into mitochondria by using an in vitro assay system (data not shown). From these combined data, we have assumed that the native ALDH5 enzyme was localized to the mitochondria.

(iv) Identification of the gene encoding commercial ALDH. The commercial enzyme was subjected to cyanogen bromide cleavage, followed by high-performance liquid chromatography purification and amino acid sequencing. Two peptides, ANDSEY and SVDALQ, were found to be identical to residues 442 to 447 and 500 to 505 encoded only by a gene located on chromosome XV (GenBank accession no. Z75282). Analysis of the DNA sequence data also suggested that this protein contained a putative leader sequence and was most likely located in the mitochondria (data not shown).

Disruption studies. (i) Identification of strains with ALDH genes disrupted. The disruptions in each of the three ALDH genes were confirmed by PCR at the DNA level (data not shown), and Western blot analysis was used to confirm the presence or absence of the individual proteins within each strain. As shown in Fig. 2, TWY397 had ALDH1, ALDH5, and ALDH2 proteins; the single-disruption strains, HWT6,

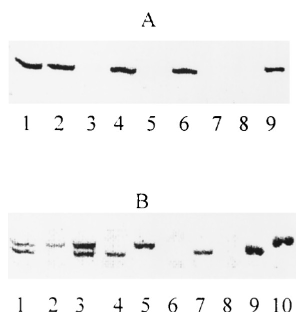

FIG. 2. Western blot analysis to identify *ALDH* disruptions. *S. cerevisiae* strains were grown on YEPD and harvested at late log phase. The cells were broken with glass beads and then centrifuged at 5,000 rpm for 10 min in a Sorvall GSA rotor. The same amount of protein from the crude lysates was loaded into each lane. The proteins were transferred to nitrocellulose membranes after electrophoresis and probed with either anti-ALDH1 (A) or anti-ALDH5 (B) antibodies. (A) Lane 1, TWY397; lane 2, HWL18; lane 3, HWT6; lane 4, HWH11; lane 5, HWTL1; lane 6, HWHL1; lane 7, HWTH2, lane 8, HWTHL10; lane 9, recombinant ALDH1. (B) Lanes 1 to 8, same as those in panel A; lane 9, recombinant ALDH5; lane 10, commercial ALDH. The subunit molecular masses of ALDH1, ALDH2, and ALDH5 were estimated to be 57, 55, and 51 kDa, respectively.

HWL18, and HWH11, were missing ALDH1, ALDH5, and ALDH2, respectively; the double-disruption strains, HWTL1, HWHL1, and HWTH2, did not have ALDH1 and ALDH5, ALDH5 and ALDH2, and ALDH1 and ALDH2, respectively; and the triple-disruption strain, HWTHL10, was missing all three ALDHs. It should be noted that antibody raised against ALDH5 also cross-reacted with ALDH2. However, the anti-ALDH5 antibody could still be used to identify disruptions of both ALDH5 and ALDH2, since the proteins migrated differently under SDS-PAGE. The anti-ALDH1 antibody had no cross-reactivity with the other two proteins.

(ii) Growth of strains with ALDH genes disrupted on glucose and ethanol. To evaluate the physiological function of each of the three ALDH proteins, the strains were grown on different medium plates containing 0.67% yeast nitrogen base in the presence of uracil, tryptophan, leucine, and histidine on 2% glucose, 2% glucose plus various concentrations of acetate, or 2% ethanol. The parent strain and strains with single ALDH gene disruptions all grew to similar colony sizes on glucose (Table 2), with the HWL18 (Δ*ald5*) strain just slightly smaller after 6 days. Only two of the double-disruption strains, HWTL1 (Δ*adl1* Δ*ald5*) and HWHL1 (Δ*ald2* Δ*ald5*), grew well on glucose. The other double-disruption strain, HWTH2 (Δ*ald1* Δ*ald2*), and the triple-disruption strain failed to grow on glucose. To test if acetate formation was blocked in the two strains which grew poorly on glucose, different concentrations of acetate (0.05, 0.1, and 0.5%) were added to 2% glucose. Addition of acetate restored the growth of HWTH2 and HWTHL10 to levels similar to those of the parent and single-disruption strains grown on glucose alone (Table 2). In our preliminary experiments, similar results were obtained when overnight cultures grown in YEPD were streaked out on plates under similar growth conditions (data not shown).

In contrast, the growth on 2% ethanol as a carbon source with a little different (Table 2). The growth of the strain with a single Δ*ald2* disruption, HWH11, was similar to that of the parent strain, TWY397, while the growth of the strain with a single Δ*ald1* disruption, HWT6, was a little slower. The strain with a single Δ*ald5* disruption, HWL18, grew very slowly on ethanol. This result contrasts with our initial experiments, which suggested that this strain could grow as well as the parent strain when streaked out on plates from a YEPD overnight culture (data not shown). The ability of this strain to grow on ethanol in earlier experiments may have been an artifact due to available glucose coming from the streaking process that allowed the cells to come out of a longer lag phase. This lag phase is even apparent when this strain is grown on 2% glucose (Table 2). The growth of all three double-disruption strains was slower than those of the parent or single-disruption strains, with the Δ*ald1* Δ*ald2* strain, HWTH2, being the slowest. The triple-disruption strain, HWTHL10, failed to grow on ethanol at all. Thus, when disruption of any of the *ALD1*, *-2*, or *-5* genes occurred, growth on ethanol was altered. Impaired growth was most pronounced when both *ALD1* and *ALD2* were simultaneously disrupted in the double- or triple-disruption strains.

Protein characterization. (i) Expression and purification of recombinant ALDH1 and ALDH5. *S. cerevisiae* ALDH1 and the mature ALDH5 without the N-terminal 23 amino acids were expressed in *E. coli*. Recombinantly expressed ALDHs

TABLE 2. Growth of yeast strains on 2% glucose (with or without 0.5% acetate) or 2% ethanol

Strain	ALDH genotype	Colony size (mm)						
		Glucose		Glucose + 0.5% acetate		Ethanol		
		Day 3	Day 6	Day 3	Day 6	Day 3	Day 6	Day 9
TWY397	*ald1 ald5 ald2*	1.0	2–2.5	1–1.5	1.5–2	1.0	1.5	1.5–2
Single disruption								
HWT6	Δ*ald1*	1.0	2.5	1.5	2–2.5	0.5–1	1.5–2	2–3
HWL18	Δ*ald5*	ND[a]	1–1.5	VS[b]	1.5	ND	ND	VS
HWH11	Δ*ald2*	0.5–1	2.5	1–1.5	1.5–2	1.0	1.5–2	2–2.5
Double disruption								
HWTH2	Δ*ald1* Δ*ald2*	ND	ND	1.0	2.0	ND	VS	0.5–1
HWHL1	Δ*ald2* Δ*ald5*	0.5–1	2–2.5	1.5	2–2.5	VS	1.5	2.0
HWTL1	Δ*ald1* Δ*ald5*	0.5–1	2.5	1.5–2	2.0	VS	1.5	2.0
Triple disruption HWTHL10	Δ*ald1* Δ*ald2* Δ*ald5*	ND	ND	2–2.5	3–4	ND	ND	ND

[a] ND, none detected (<0.01 mm).
[b] VS, very small (<0.5 mm).

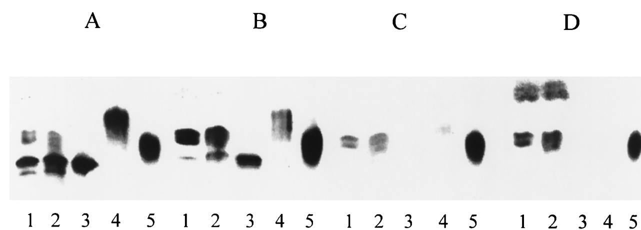

FIG. 3. IEF of the crude lysate of S288C. The cells were harvested at late log phase. Pharmalyte, pH 4.5 to 5.4, was used to make the IEF gel. After focusing, the gel was stained for activity with 5 mM NADP, 5 mM propionaldehyde, and 20 mM KCl (A); 20 mM NAD, 5 mM propionaldehyde, and 100 mM KCl (B); 5 mM NADP, 5 mM benzaldehyde, and 20 mM KCl (C); or 20 mM NADP, 5 mM benzaldehyde, and 100 mM KCl (D). Lanes 1, crude lysate grown on YEPD; lanes 2, crude lysate grown on SD; lanes 3, recombinant ALDH1; lanes 4, recombinant ALDH5; lanes 5, commercial yeast ALDH.

were purified to apparent homogeneity, as judged by Coomassie blue-stained SDS-PAGE.

The subunit molecular masses of ALDH1 and ALDH5 were estimated to be 57 and 51 kDa, respectively, as judged by SDS-PAGE. Mass spectrophotometric analysis was used to obtain more precise subunit molecular masses. It was found that the subunit molecular masses of ALDH1 and ALDH5 were 54,570.7 and 53,904.8 Da, respectively, which is within instrumental error range of the calculated values of 54,582.6 and 53,883.0 Da based on the amino acid sequences.

(ii) How many active ALDHs were present in *S. cerevisiae*? To identify active ALDH enzymes in *S. cerevisiae*, crude lysate from S288C cells was subjected to IEF agarose gel electrophoresis followed by a gel activity staining assay with either NADP or NAD and either propionaldehyde or benzaldehyde (Fig. 3). We used propionaldehyde for staining instead of acetaldehyde, due to the high evaporation rate of acetaldehyde. Five bands appeared on the gels stained with propionaldehyde and NADP in the presence of K^+ ions; their pI values were approximately 5.05, 5.15, 5.2, 5.35, and 5.4. The recombinant cytosolic ALDH1 corresponded to the major band of activity, with an estimated pI of 5.2. However, the other two bands, with pI values of 5.05 and 5.15, also reacted with anti-ALDH1 antibodies after Western blotting even though only one band appeared on SDS-PAGE, thus ruling out proteolytic degradation. These results suggested that the purified cytosolic ALDH1 preparation may have contained microheterogeneities, such as deamination of glutamine or asparagine residues, that gave rise to the different pI forms.

The recombinant yeast mitochondrial ALDH5 enzyme had a pI of 5.5. Immunoreactions showed that the ALDH5 protein was present, but no corresponding activity band was found in the crude lysate. Although the ALDH5 enzyme was present in the cells, it appears to make only a minor contribution to the overall ALDH activity (based on enzyme activity with the aldehyde substrates tested in this study). The other two bands had pI values of 5.35 and 5.4, similar to those of the commercial potassium-activated ALDH2 protein. Both ALDH1 and ALDH5 showed little activity with benzaldehyde, while the commercially available potassium-activated ALDH2 was very active. When NAD and benzaldehyde were used for the activity assay, two new bands (pI 5.9 and 6.1) appeared. The activity of cytosolic ALDH1 when assayed with NADP and propionaldehyde contributed about 70% of the total activity, as estimated by gel scanning. However, when NAD was used, the contribution of ALDH1 decreased to about 30%, with ALDH2 (the commercially available enzyme) responsible for the other 70%.

(iii) Subcellular location of *S. cerevisiae* ALDH1. After cell disruption, the cytosolic and mitochondrial fractions were subjected to IEF gel electrophoresis followed by activity staining and Western blot analysis (data not shown). The active band and the immunoreactive ALDH1 were found in the cytosolic fraction. ALDH5 was localized to the mitochondria by using a LacZ fusion protein and in vitro import experiments (data not shown). ALDH2 has been previously localized to the mitochondria (15).

(iv) Kinetic properties of purified recombinant ALDH1, ALDH5, and commercial ALDH2. The K_m value for NAD with ALDH1 was 170-fold higher than that of NADP even though the V_{max} value differed by a factor of only 2 (Table 3). Thus, we have concluded that ALDH1 is essentially an NADP-dependent enzyme. When acetaldehyde was used as the substrate, the K_m values for NADP and aldehyde were similar to those found with propionaldehyde.

The V_{max} for the *S. cerevisiae* mitochondrial ALDH5 was much lower than that of cytosolic ALDH1, and the K_m values for NADP and propionaldehyde were much higher. Mitochondrial ALDH5 could use either NAD or NADP as the cofactor and did not show a preference. When acetaldehyde was used as a substrate, the K_m for NADP decreased about fivefold and the V_{max} increased about 2.5-fold compared to the values obtained with propionaldehyde as the substrate.

Like ALDH5, the commercial ALDH2 enzyme used either NAD or NADP as a cofactor. The V_m was only twofold higher with NAD as the cofactor than it was with NADP. The V_m values for ALDH2 were two- to fourfold lower than those of

TABLE 3. Kinetic constants for recombinantly expressed *S. cerevisiae* ALDHs[a]

Enzyme	Propionaldehyde-NADP[b]			Acetaldehyde-NADP[c]			Propionaldehyde-NAD[d]		
	K_m		V_{max}	K_m		V_{max}	K_m		V_{max}
	NADP	Aldehyde		NADP	Aldehyde		NAD	Aldehyde	
ALDH1	99	30	14	40	24	24	17,400	700	8.3
ALDH5	3,470	390	0.45	640	58	1.1	6,430	830	0.011
ALDH2[e]	447	17	2.1	1,400	10	5.2	1,100	13	4.2

[a] The unit for K_m was μM, and V_{max} was presented as μmol/min/mg of protein. The assays were performed in 100 mM sodium phosphate, pH 7.4.

[b] The K_ms for NADP of cytosolic ALDH1, mitochondrial ALDH5, and commercial ALDH2 were obtained with 0.7, 5.6, and 0.28 mM propionaldehyde, respectively. The K_m values for propionaldehyde were determined with 2, 20, and 15 mM NADP.

[c] The K_ms for NADP of ALDH1, ALDH5, and ALDH2 were obtained with 0.9, 1.2, and 0.18 mM acetaldehyde, respectively. The K_m values for acetaldehyde were determined with 2, 10, and 15 mM NADP.

[d] The K_ms for NAD of ALDH1, ALDH5, and ALDH2 were obtained with 7, 5.6, and 0.28 mM propionaldehyde, respectively. The K_m values for propionaldehyde were determined with 20 mM NAD. The standard deviation was less than ±15%.

[e] The commercial yeast ALDH2 was estimated to be about 60% pure.

TABLE 4. Aldehyde activities of the three ALDHs with different aldehydes as the substrate

Enzyme	Activity (%)[a]		
	Propionaldehyde	DL-Glyceraldehyde	Benzaldehyde
ALDH1	100	12	0.9
ALDH5	100	33	2.4
ALDH2	100	68	36

[a] The activity of each ALDH with propionaldehyde as the substrate (Table 3) was assigned as 100%. The activities obtained with DL-glyceraldehyde and benzaldehyde were compared to that assayed with propionaldehyde for each ALDH. The assays were performed at V_{max} conditions.

cytosolic ALDH1. However, the commercial ALDH2 was not homogeneous. We estimated by SDS-PAGE that only about 60% of the total protein corresponded to ALDH2.

The three ALDHs could use glyceraldehyde and benzaldehyde as substrates. However, the activities were very different (Table 4). ALDH1 showed some activity with glyceraldehyde and little activity with benzaldehyde. ALDH5 had low activity with benzaldehyde but was active with glyceraldehyde. However, ALDH2 was very active with both glyceraldehyde and benzaldehyde.

(v) Metal ion effects on the activity of *S. cerevisiae* ALDHs. It has been reported that the *S. cerevisiae* mitochondrial ALDH could be activated by potassium ions (2, 3, 15, 21). It was found that the activity of ALDH5 increased maximally (about 40-fold) in the presence of 120 mM potassium chloride with NAD as the cofactor. In contrast, the enzyme activity increased only about 20-fold in the presence of 16 mM potassium chloride with NADP as the cofactor. Contrary to what was found with ALDH5, potassium chloride did not stimulate the activity of ALDH1. Recently, Dickinson (4) reported that *S. cerevisiae* cytosolic ALDH was stimulated by Mg^{2+} ions. To determine if ALDH1 was activated by Mg^{2+} ions, we tested the effects of various concentrations of divalent cations on the activity of ALDH1. We found that the activity of ALDH1 was stimulated 2.8-, 3.8-, and 2.1-fold with 20 mM $MgCl_2$, 30 mM $CaCl_2$, and 2 mM $MnCl_2$, respectively, while $ZnCl_2$ inhibited the activity of ALDH1. The concentration for 50% inhibition was 0.035 mM. The activity of commercial potassium-stimulated ALDH2 was increased fourfold in the presence of 100 mM KCl and NAD with propionaldehyde as the substrate.

DISCUSSION

In addition to ALDH1 and ALDH5, it appears from the genome sequence that five more ALDH genes are present in yeast. Amino acid sequence alignments of the cytosolic and mitochondrial ALDHs described in this study along with the five other possible ALDHs are shown in Fig. 4. The *S. cerevisiae* cytosolic ALDH1 and mitochondrial ALDH5 contained all 23 conserved amino acids found in other ALDHs (23). A gene on chromosome XV also had all 23 conserved amino acids and had an N-terminal extension with four positively charged amino acids. Amino acid sequence data showed that this protein was the commercially available mitochondrial ALDH2. Two *ALDH* genes located on chromosome XIII (*chrom13* and *chrom13i*; GenBank accession no. Z49705 and Z49700) were adjacent to each other and were almost identical (92%). It appears that they represent gene duplication. The N-terminal 20 amino acids of the two presumed duplicate proteins encoded by these genes did not have properties associated with a mitochondrial leader sequence (16, 28). A gene found on chromosome VIII (*cos8179*; GenBank accession no. U00062) would code for an ALDH-like protein, but it would be much longer than the other ALDHs (644 amino acids). Another gene on chromosome XIII (*2cos9718*; GenBank accession no. Z49702) would code for a protein with only 17 of the conserved amino acids within its 532 amino acids. None of the four proteins corresponding to these genes have been identified and studied.

All mitochondrial ALDHs have an N-terminal leader sequence needed for the import of the protein into mitochondria (16). The leader sequence usually contains 17 to 25 amino acids, has some positive charges, and can form an amphiphilic helix (28). The N-terminal amino acids of ALDH1 were not indicative of a mitochondrial leader sequence. Cell fractionation confirmed that ALDH1 was found only in the cytosolic portion. In contrast, the ALDH5 sequence had features at its N-terminal in common with a mitochondrial leader sequence. Gavel and von Heijne (6) suggested that RXY ↓ S/A is a cleavage site motif for the processing of a precursor protein. According to this cleavage pattern, we deduce that the first 23 amino acids code for a putative mitochondrial leader sequence. The experiments with ALDH5-LacZ fusion protein and in vitro import into isolated mitochondria confirmed that ALDH5 was located in mitochondria (data not shown). The first 24 amino acids of ALDH2 appeared to be similar to a typical mitochondrial leader sequence.

When *S. cerevisiae* grows on glucose, pyruvate is formed during glycolysis. Pyruvate then could be converted to acetyl-coenzyme A through two pathways. The three strains with single ALDH disruptions and the parent strain grew equally well on glucose. It appeared that either the three ALDH genes are not involved in the fermentative metabolism in a pathway generating acetyl-coenzyme A from pyruvate or the action of any one enzyme can be replaced by that of another. It was found that the double-disruption strain HWTH2 (Δ*ald1* Δ*ald2*) and the triple-disruption strain, HWTHL10 (Δ*ald1* Δ*ald2* Δ*ald5*), grew very slowly on glucose while the other two strains with double ALDH disruptions, HWTL1 (Δ*ald1* Δ*ald5*) and HWHL1 (Δ*ald2* Δ*ald5*), grew well. The slow growth on glucose suggested that both ALDH1 and ALDH2 could contribute to the formation of acetate from acetaldehyde produced in the fermentative pathway. To confirm this, 0.05, 0.1, or 0.5% acetate was added, and indeed, the growth of the double-disruption strain HWTH2 (Δ*ald1* Δ*ald2*) and the triple-disruption strain, HWTHL10 (Δ*ald1* Δ*ald2* Δ*ald5*), was restored. This suggests that acetate formation in these strains was impaired when both the *ALD1* and *ALD2* genes were missing and that the *ALD5* gene product alone could not produce enough acetate to support growth.

During fermentation, the bulk of acetaldehyde produced from pyruvate could be reduced to ethanol by alcohol dehydrogenase, resulting in ethanol accumulation. After glucose was exhausted from the medium, the accumulated ethanol could be used aerobically to serve as a sole carbon and energy source. The disrupted strains were grown on ethanol to determine the roles of *ALD1*, *ALD2*, and *ALD5*. The growth of strains with single *ALD1* (HWT6) and *ALD5* (HWL18) disruptions on ethanol was slower than that of TWY397, with the slowest growth found with HWL18. The growth on glucose and ethanol indicated that the cytosolic ALDH1 is involved in oxidation of acetaldehyde produced during fermentation and is also involved in the aerobic oxidation of acetaldehyde. The mitochondrial ALDH2 can perform these two roles as well, but ALDH1 might be more important in the oxidation of ethanol than is ALDH2. The role of mitochondrial ALDH5 has not been identified. Our in vitro enzyme kinetic data (Tables 3 and 4) suggest that ALDH5 is a relatively poor enzyme compared

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Chrom13    .......... .........M PTLYTDIEIP QLKISL.... ..KQPLGCFI NNEFC  30
Chrom13i   .......... .........M PTLYTDIEIP QLKISL.... ..KQPLGLFI NNEFC  30
Yaldh5     MLSR.TRAAA PNSRIFTRSL LRLYSQAPLR .VPITLPNGF TYEQPTGLFT NGEFV  53
Chromxv    MFSRSTLCLK TSASSIGRLQ LRYFSHLPMT .VPIKLPNGL EYEQPTGLFI NNKFV  54
Yaldh1     .......... .........M TKLHFDTAEP .VKITLPNGL TYEQPTGLFI NNKFM  35
Cos8179    IQDNQKLIGI TTLVASIFTL YVLVKIISTP AKCSSSYKPV KFSLPAPEAA QNNWK  94
2cos9718   .......... .......... .......... .......... .......... .....   0

Chrom13    PSSDGKT... .......IET VNPATGEPIT SFQAANEKDV DKAVKAARAA FDNV.  74
Chrom13i   PSSDGKT... .......IET VNPATGEPIT SFQAANEKDV DKAVKAARAA FDNV.  74
Yaldh5     ASKQKKT... .......FDV INPSNEEKIT TVYKAMEDDV DEAVAALKKL LKRSV  98
Chromxv    PSKQNKT... .......FEV INPSTEEEIC HIYEGREDDV EEAVQAADRA FSNG.  98
Yaldh1     KAQDGKT... .......YPV EDPSTENTVC EVSSATTEDV EYAIECADRA FHDTE  80
Cos8179    GKRSVSTNIW NPEEPNFIQC HCPATGQYLG SFPSKTEADI DEMVSKAGKA ....Q 145
2cos9718   .......... .......... ...MSNDGSK ILNYTPVSKI DEIVEISRNF FFEKQ  32

Chrom13    ..WS...... KTSSEQRGIY LSNLLKLIEE EQDTLAALET LDAGKPYHSN AKGDL 121
Chrom13i   ..WS...... KTSSEQRGIY LSNLLKLIEE EQDTLAALET LDAGKPFHSN AKQDL 121
Yaldh5     YCRA...... GV....RAKA LFNLADLVEK HQETLAAIES MDNGKSL.FC ARGDV 142
Chromxv    .SWN...... GIDPIDRGKA LYRLAELIEQ DKDVIASIET LDNGKAI.SS SRGDV 145
Yaldh1     ..WA...... TQDPRERGRL LSKLADELES QIDLVSSIEA LDNGKTLAFK ARGDV 127
Cos8179    STWG...... NSDFSRRLRV LASLHDYILN NQDLIARVAC RDSGKTMLDA SMGEI 194
2cos9718   LKLSHENNPR KKDLEFRQLQ LKKLYYAVKD HEEELIDAMY KDFHRNKIES VLNET  87

Chrom13    AQILQLTRYF AGSADKFDKG ATIPLTFNKF ......AYTL KVPFGVVAQI VPWNY 170
Chrom13i   AQIIELTRYY AGAVDKFNMG ETIPLTFNKF ......AYTL KVPFGVVAQI VPWNY 170
Yaldh5     ALVSKYLRSC GGWADKIY.G NVIDTGKNHF ......TYSI KEPLGVCGQI IPWNF 190
Chromxv    DLVINYLKSS AGFADKID.G RMIDTGRTHF ......SYTK RQPLGVCGQI IPWNF 193
Yaldh1     TIAINCLRDA AAYADKVN.G RTINTGDGYM ......NFTT LEPIGVCGQI IPWNF 175
Cos8179    LVTLEKIQWT IKHGQRALQP SRRPGPTNFF MKWYKGAEIR YEPLGVISSI VSWNY 249
2cos9718   TKLMNDILHL IEILPKLIKP RRVSDSSPPF M..FGKTIVE KISRGSVLII APFNF 140

Chrom13    PLAMACWKLQ GALAAGNTVI IKPAENTSLS LLYFATLIKK ....AGFPPG VVNIV 221
Chrom13i   PLAMACRKMQ GALAAGNTVI IKPAENTSLS LLYFATLIKK ....AGFPPG VVNVI 221
Yaldh5     PLLMWSWKIG PALATGNTVV LKPAETTPLS ALFASQLCQE ....AGIPAG VVNIL 241
Chromxv    PLLMWAWKIA PALVTGNTVV LKTAESTPLS ALYVSKYIPQ ....AGIPPG VINIV 244
Yaldh1     PIMMLAWKIA PALAMGNVCI LKPAAVTPLN ALYFASLCKK ....VGIPAG VVNIV 226
Cos8179    PFHNLLGPII AALFTGNAIV VKCSEQVVWS SEFFVELIRK CLEACDEDPD LVQLC 304
2cos9718   PLLLAFAPLA AALAAGNTIV LKPSELTPHT AVVMENLLTT ....AGFPDG LIQVV 191

Chrom13    ....PGYGSL VGQALASHMD IDKISFTGST KVGGFVLEAS GQSNLKDVTL ECGGK 272
Chrom13i   ....PGYGSV VGKALGTHMD IDKISFTGST KVGGSVLEAS GQSNLKDITL ECGGK 272
Yaldh5     ....PGSGRV VGERLSAHPD VKKIAFTGST ATGRHI.MKV AADTVKKVTL ELGGK 291
Chromxv    ....SGFGKI VGEAITNHPK IKKVAFTGST ATGRHI.YQS AAAGLKKVTL ELGGK 294
Yaldh1     ....PGPGRT VGAALTNDPR IRKLAFTGST EVGKSVAVDS SESNLKKITL ELGGK 277
Cos8179    YCLPPTENDD SANYFTSHPG FKHITFIGSQ PVAHYIL.KC AAKSLTPVVV ELGGK 358
2cos9718   QGAIDETTRL LD.....CGK FDLIFYTGSP RVGSIVAEK. AAKSLTPCVL ELGGK 240

Chrom13    SPALV...FE DADLDKAIDW IAA.GIFYNS GQNCTANSRV YVQSSIYDKF VEKFK 323
Chrom13i   SPALV...FE DADLDKAIEW VAN.GIFFNS GQICTANSRV YVQSSIYDKF VEKFK 323
Yaldh5     SPNIV...FA DADLDKAVKN IAF.GIFYNS GEVCCAGSRI YIQDTVYEEV LQKLK 342
Chromxv    SPNIV...FA DAELKKAVQN IIL.GIYYNS GEVCCAGSRV YVEESIYDKF IEEFK 345
Yaldh1     SAHLV...FD DANIKKTLPN LVN.GIFKNA GQICSSGSRI YVQEGIYDEL LAAFK 328
Cos8179    DAFIV...LD SAKNLDALSS IIMRGTFQSS GQNCIGIERV IVSKENYDDL VKILN 410
2cos9718   SPTFITENFK ASNIKIALKR IFF.GAFGNS GQICVSPDYL LVHKSIYPKV IK... 291

Chrom13    E.TAKKEWDV AGKFDPFDEK CIVGPVISST QYDRIKSYIE RGKREEKLDM FQTSE 377
Chrom13i   E.TAKKEWDV AGKFDPFDEK CIVGPVISST QYDRIKSYIE RGKKEEKLDM FQTSE 377
Yaldh5     D.YTES.LKV G...DPFDEE VFQGAQTSDK QLHKILDYVD VAKSEG..AR LVTGG 390
Chromxv    A.ASES.IKV G...DPFDES TFQGAQTSQM QLNKILKYVD IGKNEG..AT LITGG 393
Yaldh1     A.YLETEIKV G...NPFDKA NFQGAITNRQ QFDTIMNYID IGKKEG..AK ILTGG 377
Cos8179    DRMTANPLRQ GSDIDHLENV DM.GAMISDN RFDELEALVK DAVAKG..AR LLQGG 462
2cos9718   ....ECESVL NEFYPSFDEQ TDFTRMIHEP AYKKAVASIN STNG....SK IVPSK 338

Chrom13    FPIGGAK... GYFIPPTIFT DVPQTSKLLQ DEIFGPVVVV SKFTNYDDAL .KLAN 428
Chrom13i   FPIGGAK... GYFIPPTIFT DVPETSKLLR DEIFGPVVVV SKFTNYDDAL .KLAN 428
Yaldh5     ARHGS.K... GYFVKPTVFA DVKEDMRIVK EEVFGPIVTV SKFSTVDEVI .AMAN 440
Chromxv    ERLGS.K... GYFIKPTVFG DVKEDMRIVK EEIFGPVVTV TKFKSADEVI .NMAN 443
Yaldh1     EKVGD.K... GYFIRPTVFY DVNEDMRIVK EEIFGPVVTV AKFKTLEEGV .EMAN 427
Cos8179    SRFKHPKYPQ GHYFQPTLLV DVTPEMKIAQ NEVFGPILVM MKAKNTDHCV .QLAN 516
2cos9718   ISINSDTEDL .CLVPPTIVY NIGWDDPLMK QENFAPVLPI IEYEDLDETI NKIIE 392

Chrom13    DTCYGLASAV FTKDVKKAHM FARDIKAGTV WI.NQSNDED V.TVPFGGFK MSGIG 481
Chrom13i   DTCYGLASAV FTKDVKKAHM FARDIKAGTV WI.NQTNQEE A.KVPFGGFK MSGIG 481
Yaldh5     DSQYGLAAGI HTNDINKAVD VSKRVKAGTV WI.NTYNNFH Q.NVPFGGFG QSGIG 493
Chromxv    DSEYGLAAGI HTSNINTALK VADRVNAGTV WI.NTYNDFH H.AVPFGGFN ASGLG 496
Yaldh1     SSEFGLGSGI ETESLSTGLK VAKMLKAGTV WI.NTYNDFD S.RVPFGGVK QSGYG 480
Cos8179    SAPFGLGGSV FGADIKECNY VANSLQTGNV AI.NDFATFY VCQLPFGGIN GSGYG 570
2cos9718   EHDTPLVQYI FSDSQTEINR ILTRLRSGDC VVGDTVIHVG ITDAPFGGIG TSGYG 447

Chrom13    RELGDNVVDT YLQTKSVHMD LSLDN..... .......... .......... ..... 506
Chrom13i   RESGDTGVDN YLQIKSVHVD LSLDK..... .......... .......... ..... 506
Yaldh5     REMGEAALSN YTQTKSVRIA IDKPIR.... .......... .......... ..... 519
Chromxv    REMSVDALQN YLQVKAVRAK LDE....... .......... .......... ..... 519
Yaldh1     REMGEEVYHA YTEVKAVRIK L......... .......... .......... ..... 501
Cos8179    KFGGEEGLLG LCNAKSVCFD TLPFVSTQIP KPLDYPIRNN AKAWNFVKSF IVGAY 625
2cos9718   NYGGYYGFNT FSHERTIFKQ PYWNDFTLFM RYPPNSAQKE KLVRFAMERK PWFDR 502
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FIG. 4. Amino acid sequence alignments of seven possible ALDHs in *S. cerevisiae*. Yaldh1, cytosolic ALDH1; Yaldh5, mitochondrial ALDH5; Chromxv, commercial ALDH2, encoded by *ALD2*, located on chromosome XV; Chrom13 and Chrom13i, two adjacent potential ALDHs encoded by genes located on chromosome XIII. Cos8179 is a potential ALDH encoded by a gene located on chromosome VIII; it would consist of 644 amino acids. Neither the first 39 amino acids (MSKVYLNSDMINHLNSTVQAYFNLWLEKQNAIMRSQPQI) at the N terminal nor the last 19 amino acids (TNSTWQRIKSLFSLAKEAS) at the C terminal are shown. 2cos9718 is a potential ALDH also encoded by a gene located on chromosome XIII and sequenced from cosmid 9718; it would contain 532 amino acids. The last 30 amino acids (NGNNKWGLRQYFSLSAAVILISTIYAHCSS) at the C terminal are not shown. The underlined residues are those conserved in all known ALDHs. The sequences for ALDH1 and ALDH5 were from this study, while the others were from GenBank.

to the other two. This is also reflected in our in vivo data for the strain with a single *ALD5* disruption grown on ethanol (Table 2).

Even though seven genes in *S. cerevisiae* could encode ALDH-like proteins, how many of these genes are actually expressed in *S. cerevisiae* is not known. Three different pI forms of ALDH1 were found after IEF. Only one band appeared on the SDS-polyacrylamide gels, indicating that these forms were not proteolytic products. These could result from deamination, since after ALDH1 gene disruption, those three bands were absent. The enzyme activities of both ALDH5 and the commercially available yeast ALDH2 were stimulated by potassium. However, they had different pI values and exhibited different staining properties, with the commercial ALDH showing high activity with benzaldehyde while ALDH5 had low activity. A completely different band of activity, with a pI value of 5.9, was identified when benzaldehyde and NAD were used in the activity staining. The other three ALDH gene products might be expressed in *S. cerevisiae*, but if they were, their activities were too low to be detected under the assay conditions employed in this study or their substrate specificities were very different from those of the other enzymes.

The kinetic properties of the *S. cerevisiae* cytosolic ALDH1 were similar to those of the cytosolic enzyme found by Seegmiller in 1953 (20) and recently studied by Dickinson (4). Seegmiller reported that the K_ms for NADP and acetaldehyde were 14 and 34 μM, respectively, while Dickinson found that K_ms for NADP and acetaldehyde were 66 and 100 μM, respectively. The K_ms for NADP and acetaldehyde of the recombinantly expressed ALDH1 in this study were found to be 99 and 24 μM, respectively. The recombinantly expressed ALDH1 was also activated by Mg^{2+} ions, consistent with the data reported by Seegmiller (20) and Dickinson (4). Thus, it appears that we established the relationship between the sequence and the kinetic properties of a major *S. cerevisiae* cytosolic ALDH.

Black (2, 3) found a potassium-activated ALDH in yeast. Jacobson and Bernofsky (15) found that this ALDH was located in mitochondria. The recombinant mitochondrial ALDH5 is potassium stimulated and used both NAD and NADP as the cofactor, consistent with what was found by previous investigators. Black reported that the K_ms for NAD and acetaldehyde were 30 and 80 μM, respectively. Steinman and Jakoby (21) also found that the potassium-stimulated ALDH had low K_ms for NAD, NADP, and acetaldehyde (20, 50, and 3 μM, respectively). However, in this study, we found that the mitochondrial ALDH5 had much higher K_ms for NAD(P) and aldehyde. Furthermore, investigators reported that the mitochondrial potassium-stimulated ALDH was very active with benzaldehyde (2, 3) and was repressed 99% by glucose (15), while we found that the mitochondrial ALDH5 had low activity with benzaldehyde and most likely was a constitutive enzyme. The properties reported by others were more similar to those we found to be associated with the commercially available K^+-activated ALDH2, which is encoded by a gene on chromosome XV.

Investigators previously reported that *S. cerevisiae* cytosolic ALDH was active exclusively with NADP and no dehydrogenase activity was observed with NAD as the cofactor (20). It was found that the recombinant *S. cerevisiae* cytosolic ALDH1 was active with NAD. However, the K_m value for NAD was more than 170 times higher than that of NADP and the enzyme activity, like that of mammalian ALDH1, was inhibited at very high concentrations of NAD (29). The investigators usually considered that all dehydrogenase activity was due to *S. cerevisiae* mitochondrial ALDH when they used NAD as the cofactor (17). When NAD is used for dehydrogenase assay, the contribution of the cytosolic ALDH1 to the total activity cannot be excluded, as indicated by activity staining of IEF gels.

ALDH1 is present at a higher level in the cells than ALDH5, as estimated by SDS-PAGE followed by Western blot analysis. Thus, their kinetic properties in vitro might provide insight into their functions in ethanol metabolism in vivo. To investigate the possible roles in ethanol metabolism of ALDHs in yeast, S288C grown on either YEPD or SD was harvested at late log phase, in which accumulated ethanol was used as a carbon source. When assayed in the presence of K^+ ions, the contribution of ALDH1 to the total ALDH activity was estimated to be between 30 and 70% (data not shown), depending on the cofactor used. This showed that ALDH1 contributes to ethanol metabolism, provided that NADP is available. It appeared that acetaldehyde oxidation in the cytosol can range from 70 to 30% depending on the NADP/NAD ratio. The contribution of ALDH2 was only about 30% with NADP. However, the contribution was increased to about 70% when NAD was used for staining. Thus, it appeared that ALDH2 was a major mitochondrial ALDH. Its contribution to the oxidation of acetaldehyde, like that of ALDH1, may depend on the NADP/NAD ratio.

The kinetic properties of *S. cerevisiae* ALDH enzymes were different from those of mammalian ALDHs. The dehydrogenase activities of mammalian mitochondrial ALDHs were enhanced about twofold by Mg^{2+}, Mn^{2+}, and Ca^{2+} ions, while the cytosolic ALDH1 was inhibited by those divalent ions (23, 24, 26, 27, 32). In contrast to what was found with mammalian liver enzymes, the activity of *S. cerevisiae* cytosolic ALDH1 was stimulated, not inhibited, by Mg^{2+}, Mn^{2+}, and Ca^{2+} ions while yeast mitochondrial ALDHs were stimulated by K^+ ions. In mammals, mitochondrial ALDH2, rather than cytosolic ALDH1, is the major enzyme responsible for ethanol metabolism (22). *S. cerevisiae* cytosolic ALDH1 appears to play a major role in acetaldehyde oxidation.

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