Functional analysis of the ALD gene family of Saccharomyces cerevisiae during anaerobic growth on glucose: the NADP⁺-dependent Ald6p and Ald5p isoforms play a major role in acetate formation

Florence Saint-Prix, Linda Bönquist and Sylvie Dequin

In Saccharomyces cerevisiae, acetate is formed by acetaldehyde dehydrogenase (ACDH), a key enzyme of the pyruvate dehydrogenase (PDH) bypass, which fulfils the essential task of generating acetyl-CoA in the cytosol. The role of the five members of the ACDH family (ALD genes) was investigated during anaerobic growth on glucose. Single and multiple aldΔ mutants were generated in the wine-yeast-derived V5 and laboratory CEN.PK strains and analysed under standard (YPD 5% glucose) and wine (MS 20% glucose) fermentation conditions. The deletion of ALD6 and ALD5 decreased acetate formation in both strains, demonstrating for the first time that the mitochondrial Ald5p isoform is involved in the biosynthesis of acetate during anaerobic growth on glucose. Acetate production of the ald4Δ mutant was slightly decreased in the CEN.PK strain during growth on YPD only. In contrast, the deletion of ALD2 or ALD3 had no effect on acetate production. The absence of Ald6p was compensated by the mitochondrial isoforms and this involves the transcriptional activation of ALD4. Consistent with this, growth retardation was observed in ald6Δald4Δ, and this effect was amplified by the additional deletion of ALD5. A aldΔ null mutant, devoid of ACDH activity, was viable and produced similar levels of acetate to the ald6Δald4Δald5Δ strain, excluding a role of Ald2p and Ald3p. Thus, acetate is mainly produced by the cytosolic PDH bypass via Ald6p and by a mitochondrial route involving Ald5p. An unknown alternative pathway can compensate for the loss of Ald6p, Ald4p and Ald5p.

INTRODUCTION

In Saccharomyces cerevisiae, acetate is produced as an intermediate of the pyruvate dehydrogenase (PDH) bypass, which converts pyruvate into acetyl-CoA in a series of reactions catalysed by pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ACDH) and acetyl-CoA synthetase. This pathway is the sole source of cytosolic acetyl-CoA, which is required for anabolic processes such as lipid biosynthesis (Flikweert et al., 1996; Pronk et al., 1996). The reaction catalysed by ACDH also generates reducing equivalents, which are required for a variety of synthetic pathways and redox reactions, in the form of NAD(P)H. ACDH produces acetate by oxidizing the acetaldehyde produced from pyruvate during the fermentation of sugars and that formed during ethanol oxidation.

Two ACDHs were originally identified in S. cerevisiae: an Mg²⁺-activated, NADP⁺-dependent cytosolic enzyme (Seegmiller, 1953) and a glucose-repressed, NAD(P)⁺-dependent, mitochondrial enzyme activated by K⁺ and thiols (Jacobson & Bernofsky, 1974). Five ACDH genes have now been identified in the genome sequence of S. cerevisiae S288C. According to the nomenclature of Navarro-Avino et al. (1999), the cytosolic ACDHs are encoded by ALD6 (YPL061w), ALD2 (YMR170c) and ALD3 (YMR169c), whereas the mitochondrial isoforms are encoded by ALD4 (YOR374w) and ALD5 (YER073w). Ald6p and Ald4p, which are Mg²⁺- and K⁺-dependent, respectively, are the major isoforms and have been shown to be involved in growth on glucose and on ethanol (Meaden et al., 1997; Tessier et al., 1998; Wang et al., 1998). However, the behaviour of deletion mutants indicates that the cytosolic and mitochondrial isoforms may, in some conditions, be at least partially redundant (Boubekeur et al., 1999, 2001; Remize et al., 2000). We reported previously that a double mutant, ald6Δald4Δ, produced acetate during anaerobic growth on glucose, indicating that other routes exist for the synthesis of cytosolic acetyl CoA (Remize et al., 2000). The roles of the minor cytosolic and mitochondrial

Abbreviations: ACDH, acetaldehyde dehydrogenase; HA, haemagglutinin; PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase.
isozymes of ACDH in acetaldehyde metabolism have not been clearly defined. Ald2p and Ald3p are encoded by tandem reading frames, which display 92% identity. ALD2 and ALD3 are induced by a variety of stresses (Navarro-Avino et al., 1999; Norbeck & Blomberg, 2000) and it has been suggested that these NAD+–dependent isozymes play a role in redox metabolism, particularly under conditions of osmotic stress (Norbeck & Blomberg, 1997, 2000; Akhtar et al., 1997). However, the only detectable phenotype of the ald2Δald3Δ double mutation is a lower than normal growth rate on ethanol, suggesting a possible role for the corresponding isozymes in ethanol oxidation (Navarro-Avino et al., 1999). Ald5p makes only a minor contribution to total ACDH activity (Navarro-Avino et al., 1999). It has been suggested that this isoform is involved in respiratory metabolism, based on the low levels of cytochrome in an ald5Δ mutant (Kurita & Nishida, 1999).

The objective of this work was to investigate the specific contribution of each Aldp isozyme to acetate production during anaerobic glucose fermentation. This study, in addition to providing new insight into acetaldehyde metabolism, also has important implications for industrial yeast-based processes, because the acetate produced by the PDH bypass is the most abundant organic acid accumulating during the alcoholic fermentation of sugars.

We constructed a set of single and multiple aldΔ mutants in two S. cerevisiae strains with different genetic backgrounds (a laboratory and a wine-yeast-derived strain). We analysed the growth and acetate production of the deletion mutants during anaerobic fermentation, under standard (YPD 5% glucose) or wine fermentation (MS 20% glucose) conditions. Wine fermentation conditions typically involve multiple stresses (osmotic, acidic and ethanol stress, nitrogen limitation). We provide evidence that Ald6p, Ald5p and, depending on strain and culture conditions, Ald4p are required for acetate formation during anaerobic growth on glucose, whereas Ald2p and Ald3p are not. We also show that the mitochondrial PDH bypass can compensate for the absence of Ald6p and that this compensation involves the transcriptional activation of ALD4. We also report that mutants devoid of ACDH activity are viable, indicating that an unknown, alternative pathway produces acetate, and thus cytosolic acetyl-CoA, in the mutants.

**METHODS**

**Strains and media.** The strains used are shown in Table 1. The V5 strain, used for expression studies and to construct the aldΔ

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tr>
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<td>MATa ura3</td>
<td>INRA UMR SPO</td>
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<tr>
<td>V5 ald6Δ</td>
<td>MATa ura3 ald6Δ::loxP</td>
<td>This study</td>
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<tr>
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<td>MATa ura3 ald5Δ::loxP</td>
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<tr>
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<td>E. Boles</td>
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<td>This study</td>
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*INRA UMR SPO, UMR Sciences pour l’Enologie, Microbiologie et Technologie des Fermentations, INRA, Montpellier, France; E. Boles, Institut für Mikrobiologie, Frankfurt, Germany.
mutants, was derived from a Champagne strain and has fermentation characteristics similar to those of industrial wine yeast strains. The CEN.PK2-1C strain was used as a laboratory parental strain for the construction of aldΔ mutants, and the isogenic strain CEN.PK2-1D was also used to produce the aldΔ mutant. Strains were cultured and maintained on YPD, containing 10 g yeast extract l\(^{-1}\), 20 g peptone l\(^{-1}\) and 20 g glucose l\(^{-1}\). Sporulation medium contained 1 g yeast extract l\(^{-1}\), 0.5 g glucose l\(^{-1}\), 10 g potassium acetate l\(^{-1}\) and 20 g agar l\(^{-1}\).

**Fermentation media and conditions.** Batch fermentation experiments were carried out on YPD containing 5% glucose or on synthetic MS medium. MS medium, which is similar in composition to grape must, contains 15–20% glucose, 6 g malic acid l\(^{-1}\), 6 g citric acid l\(^{-1}\) and 300 mg nitrogen l\(^{-1}\) in the form of amino acids (180 mg N l\(^{-1}\)) and NH\(_4\)Cl (120 mg N l\(^{-1}\)), pH 3.3 (Bely et al., 1990). MS medium was supplemented with 115 mg methionine l\(^{-1}\) and 50 mg uracil l\(^{-1}\) for the V5-derived strains, and with appropriate amino acids for the CEN.PK-derived strains. Ergosterol (7.3 mg l\(^{-1}\)), oleic acid (2.5 mg l\(^{-1}\)) and Tween 80 (0.21 g l\(^{-1}\)) were provided as anaerobic growth factors.

Cells were first cultured in Erlenmeyer flasks containing 20 ml MS or YPD medium for 30 and 24 h, respectively, at 28 °C with shaking. Fermenters (0-25 l), filled to 80% of their volume, were inoculated with cells from these precultures at a density of 1 × 10\(^{6}\) cells ml\(^{-1}\) and fermentation was carried out at 28 °C with continuous stirring (500 r.p.m.). The carbon dioxide produced by the fermentation reaction was evacuated from the fermenter via a thin glass tube that passed through the cork used to seal the fermenter. Fermentation experiments were performed in triplicate and one representative experiment is shown.

**Construction of aldΔ mutants in the V5 and CEN.PK strains.** Genes were deleted by the short flanking homology method using the loxP-kanMX4-loxP gene disruption cassette and the Cre-Lox recombination system, which allows marker recycling (Guldener et al., 1996). The deletion cassette was amplified from pUG6 (Guldener et al., 1996) using the primer pairs listed in Table 2 for each ALD gene. The forward primer has 18 nt (bold type) complementary to the sequence of pUG6 and an extension of 36–42 nt complementary to pUG6-HA (bold type) and nucleotide extensions and downstream from the ALD integration of the replacement cassettes was confirmed by PCR on this study, we found that V5 also possesses two copies of *S. cerevisiae* and the published PCR-based gene disruption method (Guldener et al., 1996). A PCR fragment carrying three consecutive copies of the 27 bp sequence encoding the HA epitope and the loxP-kanMX4-loxP cassette was amplified from pUG6-HA (Makuc et al., 2001) using the primers listed in Table 2. These primers have stretches of 18 and 22 nt, respectively, complementary to pUG6-HA (bold type) and nucleotide extensions to direct integration and to replace the stop codon of each *ALD* gene. Correct integration of the replacement cassettes was confirmed by PCR on genomic DNA using primers complementary to regions upstream and downstream from the *ALD* ORF. The kanMX module was removed from the genome using pSH47 before Western blot analysis.

**RNA extraction and Northern blot analyses.** Total RNA was isolated from 2 × 10\(^8\) cells using Trizol reagent (Gibco-BRL, Life Technologies), as described previously (Remize et al., 2001). RNA samples (15 μg per lane) were separated by electrophoresis in 1% agarose gels containing formaldehyde, blotted by capillary transfer onto Hybond-N\(^+\) membranes (Amersham) as described by Sambrook et al. (1989) and cross-linked by exposure to low-wavelength UV radiation for 1 min. Membranes were prehybridized by incubation for 2–4 h at 50 °C in 0.5 M phosphate buffer (pH 7), 1 mM EDTA, 7% SDS and 1% BSA. Membranes were hybridized with \(^{32}\)P-labeled oligonucleotides (2 × 10\(^4\) cp.m. ml\(^{-1}\)) used as probes, in the same solution, at 50 °C for 18 h. The membranes were washed twice in 2-5 mM phosphate buffer (pH 7), 6 × SSC, 0.25% SDS for 5 min at room temperature and twice for 5 min at the hybridization temperature, and analysed with a PhosphorImager (Molecular Dynamics). Probes were prepared by labelling oligonucleotides (Table 2) with \([\gamma-^{32}\text{P}]\text{ATP}\) using T4 polynucleotide kinase (Promega) and purified using a silica-gel membrane (QIAquick Nucleotide Removal; Qiagen). The specificities of the oligonucleotides used to probe *ALD* genes were checked by hybridization to the chromosomal sequences of the corresponding *aldΔ* mutants. S25 RNA was used as a loading control and was detected by probing the membrane with the oligonucleotide 5’- CCTCCGCTTATTGATATGCTTAAAG-3’.

**Crude cell extract preparation and Western blot analyses.** Yeast cells (1 × 10\(^8\) cells) were harvested by centrifugation, washed in 9 g NaCl l\(^{-1}\) and resuspended in 0.5 ml 100 mM phosphate buffer (pH 7.6). Cells were disrupted with glass beads and centrifuged. The supernatant was used as crude extract. The total protein concentration of the crude extract was determined by the method of Bradford (1976) using a Bio-Rad kit and BSA as the standard. HA-tagged proteins were analysed by SDS-PAGE followed by transfer onto Hybond-C Extra membranes (Amersham) as described by Sambrook et al. (1989). The gels used for SDS-PAGE contained 4.5% acrylamide in the stacking gel and 10% acrylamide in the resolving gel. Membranes were then incubated for 30 min at room temperature with 1% BSA in 20 mM Tris/HCl, 150 mM NaCl and 0-05% Tween 20 (pH 7.5) for blocking. The blots were incubated overnight at room temperature with the anti-HA mAb (Sigma) at a final dilution of 1:5000, washed and incubated for 30 min at room temperature with anti-mouse IgG alkaline phosphatase conjugate (Promega). The blots were washed and antibody binding was detected by incubation with Western Blue stabilized substrate for alkaline phosphatase (Promega), according to the manufacturer's instructions.

**Analytical methods.** Optical density was measured at 660 nm. Acetic acid was determined by HPLC using an HPX-87H ion exclusion column (Bio-Rad) and by enzymic assays (Boehringer detection kit). Glucose was determined by a colorimetric method using 3,5-dinitrosalicylic acid as described by Miller (1959).

**Cell extracts and enzyme assays.** Cell extracts were obtained as described previously (Remize et al., 2000). Enzyme activities were assayed immediately after the preparation of cell extracts. Protein
concentration was determined by the method of Bradford (1976) using a Bio-Rad kit and BSA as the standard. For the determination of total ACDH specific activity, the reaction was carried out as described by Postma et al. (1989) except that we added MgCl₂ (10 mM) and both NAD⁺ and NADP⁺ to the reaction mixture.

RESULTS

Ald6p and Ald5p are the major isoforms required for acetate formation

We first analysed the expression of ALD genes in the wine-yeast-derived V5 strain during fermentation on MS (20 % glucose). Wine fermentation involves a short exponential growth phase since nitrogen is limiting, followed by a stationary phase, during which most of the sugar is consumed. About 80% of all the acetate produced is generated during the first 40 h (Fig. 1a). Changes in ALD mRNA and protein levels during the course of fermentation were investigated by Northern blotting with gene-specific probes and by Western blotting with strains expressing HA-tagged ALD genes, respectively. Fig. 1 shows the mRNA profiles, relative mRNA expression (Fig. 1b) and the protein profiles (Fig. 1c) during wine fermentation. The Northern data indicated a very low expression level of ALD2 and ALD3 during wine fermentation compared to other ALD genes.
Fig. 1. Expression of *ALD* genes in the V5 strain during fermentation on MS medium. (a) Growth (filled triangles) and acetate (open triangles) production. (b) Northern blot analysis. Total RNA was extracted from V5 cells collected at the fermentation times indicated. Total RNA (15 μg per lane) was separated by electrophoresis and subjected to Northern blotting using specific oligonucleotides as probes. The relative mRNA expression was calculated by normalization to the S25 signal. (c) Western blot analysis. Crude extracts were prepared from strains carrying an *ALD* gene encoding an HA epitope at its 3' end. The *ALD::HA* strains were cultured on MS medium and the cells were collected at the times indicated. Total proteins (5 μg per lane) were separated by SDS-PAGE and subjected to Western blot analysis using anti-HA mAb.
Transcripts were detected during the growth phase, whereas the corresponding proteins were detected at the end of exponential growth and during the stationary phase. It is unlikely that the discrepancies between transcripts and proteins might be due to the fact that these data come from different fermentation experiments since each fermentation with HA protein was identical (growth, biomass, fermentation duration) to that on which Northern analysis was performed (Fig. 1a). Rather, the level of mRNA for these two genes might be too low to determine accurate variations in transcript level. Protein data seem therefore to be a better indicator of ALD2 and ALD3 expression. The other three genes are expressed at a higher level. For these genes, proteins start to be detected at about the same time as mRNA, at the beginning of fermentation for ALD6 and ALD5 and in the late exponential phase (around 20 h) for ALD4. Some differences were observed between the transcripts and protein profiles. In particular, the protein levels did not vary as for the mRNA levels, but rather appeared constant. This might be due to differences in detection sensitivity of Northern and Western assays and/or small variations in time points for mRNA and protein level. The fact that the proteins stayed at a high level throughout the fermentation whereas a strong decline in mRNA levels was observed when the cells entered the stationary phase, indicates a high stability of Aldp proteins during the enological stationary phase. As a whole, these data show that during the exponential phase, where the synthesis of acetate, precursor of acetyl-CoA, might be critical for growth, Ald6p and Ald5p were the major isoforms detected.

We assessed the relative contributions of the various ACDH isoforms during fermentation by examining the impact of deleting each gene on growth and acetate production. We deleted the full-length coding sequences of ALD2–6 and replaced them with the kanMX4 marker in the wine-yeast-derived strain V5 and in the laboratory strain CEN.PK2-1C. We studied the single deletion mutants during anaerobic fermentation on YPD containing 5 % glucose and in synthetic MS medium. The laboratory strain grew poorly on MS medium and the fermentation stopped before glucose was exhausted (data not shown). Decreasing the initial sugar concentration from 200 to 150 g l⁻¹ resulted in complete fermentation. The CEN.PK series was therefore analysed on MS containing only 15 % glucose. Marked differences were observed between the two strains on MS medium. It is well known that laboratory strains are poorly adapted to wine fermentation conditions. Under these multiple stress conditions (high sugar, low pH, low nitrogen), the V5 strain fermented sugars rapidly, whereas the duration of fermentation was considerably longer for the CEN.PK strain. The latter strain also formed lower biomass levels and considerably higher acetate levels than the V5 strain (Table 3). In contrast, the two strains showed similar behaviour for growth and acetate production on YPD medium. Under the various growth conditions used (YPD, MS), the growth rate and final biomass of the single aldΔ mutants were similar to those of wild-type strains (data not shown). In contrast, marked differences in acetate production were observed (Table 3). Consistent with the reported major role of ALD6 in acetate biosynthesis on glucose (Meaden et al., 1997; Remize et al., 2000), the deletion of ALD6 markedly reduced acetate production. The wild-type produced 2 and 3.3 times more acetate than the V5 aldΔ mutant in MS and YPD medium, respectively, and 1.2 and 4 times more acetate than the CEN.PK aldΔ mutant in MS and YPD medium, respectively. It has been suggested that both Ald6p and Ald4p might catalyse the oxidation of acetaldehyde produced during fermentation (Wang et al., 1998). We found that ALD4 deletion did not significantly affect acetate production in the V5 strain and slightly decreased the level of acetate formed by CEN.PK in YPD only (14 %), indicating that the contribution of ALD4 to acetate production during fermentation depends on strain and culture conditions. Surprisingly, the deletion of ALD5 reduced acetate formation by 24 and 22 % in the V5 strain and by 26 and 22 % in the CEN.PK strain in MS and YPD, respectively. Thus, the Ald5p isoform is also involved in acetate biosynthesis. In contrast, the ald2Δ and ald3Δ mutants produced similar amounts of acetate to the wild-type, indicating that Ald2p and Ald3p play no role in acetate biosynthesis under these conditions. The precise contributions of Ald6p, Ald5p and Ald4p to acetate production depended on the composition of the medium. In particular, the effect of the ALD6 deletion in both strains and that of ALD4 in the CEN.PK background were more marked on YPD medium than on MS medium, whereas the mitochondrial Ald5p isoform made a similar contribution to acetate synthesis in all conditions.

**Table 3. Acetate production of single aldΔ mutants in V5 and CEN.PK strains after fermentation on MS medium with 15–20 % glucose or on YPD with 5 % glucose**

<table>
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<th>Acetate concentration (g l⁻¹)</th>
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<tr>
<td></td>
<td>V5</td>
</tr>
<tr>
<td></td>
<td>MS 20 %</td>
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<tr>
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</tr>
<tr>
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<tr>
<td>ald2Δ</td>
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</table>

We investigated the impact of the double deletions aldlΔald5Δ and aldlΔald4Δ during glucose fermentation. Double deletion of ALD6 and ALD5 in strain V5 (Fig. 2a) or CEN.PK (Fig. 2b) did not affect growth. Acetate production was reduced by 77 % in strain V5 aldlΔald5Δ grown
on MS medium, showing a cumulative effect of each deletion (Fig. 2a). Deletion of ALD5 in the CEN.PK ald6 mutant did not further reduce acetate production during growth on YPD (Fig. 2b). However, the ald6 mutant produces very low acetate levels under such conditions and small variations resulting from ALD5 deletion might have been undetectable.

Although the ald6Δ and ald4Δ mutants grew normally, double deletion of ALD6 and ALD4 in both strains decreased the growth rate on MS medium (Fig. 2). This effect was previously observed in the V5 background (Remize et al., 2000). The production of acetate was affected in the V5 ald6Δald4Δ mutant, in agreement with the decreased growth rate. However, the final level of acetate produced was similar to that of ald6Δ mutants. The phenotype of the ald6Δald4Δ mutants strongly suggests that Ald4p compensates for the loss of Ald6p. We therefore investigated the possibility that ALD4 or another ALD gene was transcriptionally activated, by analysing changes in ALD mRNA levels in strain V5 and V5 ald6Δ strains during fermentation. We also determined Ald4p and Ald5p levels in V5 and V5 ald6Δ strains carrying ALD4::HA or ALD5::HA alleles. Levels of ALD2, ALD3 and ALD5 mRNA, and levels of Ald5 protein in the ald6Δ mutant were similar to those in the wild-type strain (Fig. 3). In contrast, ALD4 transcripts were detected earlier in the fermentation for the ald6Δ mutant than for the wild-type. ALD4 was not expressed during the first 20 h in strain V5 (Figs 1b and 3a), whereas both ALD4 mRNA (Fig. 3a) and protein (Fig. 3b) were detected in large amounts during the growth phase in the mutant, thereby compensating for the loss of Ald6p. We also found that the ALD4 mRNA level in the CEN.PK ald6Δ mutant was twofold higher than in the wild-type strain during exponential growth in YPD medium (data not shown).

**ALD2 and ALD3 cannot compensate for the loss of ALD6, ALD4 and ALD5**

The double ald6Δald4Δ V5 and CEN.PK mutants produced significant levels of acetate. This suggests that some of the remaining ALD genes are sufficiently active to support acetate production or alternatively that they are activated to compensate for the loss of Ald6p and Ald4p. We investigated these possibilities by comparing transcript levels for ALD2, ALD3 and ALD5 in the V5 double mutant and in the wild-type strain. No significant differences were observed between the two strains in the level and pattern of expression of these genes during fermentation (data not shown). This suggests that basal expression of the remaining genes is sufficient for the production of enough acetate to support

**Fig. 2.** Impact of the deletion of ALD4 in ald6Δ mutants. (a) Growth and acetate production of V5 (filled triangles) and ald6Δ (open squares), ald4Δ (open diamonds), ald5Δ (filled diamonds), ald6Δald4Δ (+) and ald6Δald5Δ (filled circles) mutants on MS medium. (b) Growth and acetate production of CEN.PK (filled triangles) and ald6Δ (open squares), ald4Δ (open diamonds), ald5Δ (filled diamonds), ald6Δald4Δ (+) and ald6Δald5Δ (filled circles) mutants on YPD 5% glucose medium. Acetate was determined after complete sugar exhaustion at 48 h for the ald6Δald4Δ mutant and 28 h for all other strains.
growth. We tried to determine which genes were active in the double mutant by deleting ALD5 or both ALD3 and ALD2 in this mutant. The quadruple ald6Dald4Dald3Dald2D mutant behaved identically to the ald6Dald4D mutant (Fig. 4), suggesting that the two cytosolic isoforms are not required for acetate synthesis. In contrast, the deletion of ALD5 from the double mutant strongly impaired growth, considerably delaying acetate production. The ald6Dald4Dald5D triple mutant displayed a prolonged lag phase of about 40 h, after which the mutant began to grow. However, total biomass levels for this mutant were slightly lower than those for the wild-type. Acetate production was detected after about 80 h. Fermentation was complete after about 160 h in this mutant, and after 90 h in the wild-type V5 strain. The CEN.PK triple mutant also exhibited a growth defect compared to the ald6Dald4D mutant on YPD containing 5% glucose or MS containing 15% glucose (data not shown). The double and triple mutants displayed normal growth in YPD medium supplemented with acetate (data not shown), indicating that the growth defects of these mutants resulted from an inability to produce acetate at the beginning of fermentation. These results suggest that Ald5p is required for acetate formation during fermentation, consistent with the results presented above.

As the ald6Dald4Dald5D triple mutant produced acetate, we investigated whether Ald2p and Ald3p compensated for the loss of the three main isoforms. We produced a strain in which ALD2–6 (ald4D mutant) were deleted by crossing strain CEN.PK2-1C ald6Dald4Dald3ald2D and strain CEN.PK2-1D ald5D::kanMX and isolating spores on YPD medium supplemented with acetate. The deletion of each ALD gene was verified in these three strains by PCR analysis. As shown for one spore in Fig. 5(a), all ALD genes were deleted. Similar results were obtained for the two other strains. The deletion of ALD5 in the three ald4D mutants was also confirmed by Southern blotting of genomic DNA digested by EcoRV. As expected, a fragment...
of correct size was detected in the control strain but not in the three aldΔ mutants (data not shown).

During fermentation on YPD containing 5% glucose or MS containing 15% glucose, the phenotype of the null mutant was similar to that of the ald6Δald4Δald5Δ mutant, in terms of both growth and acetate production (Fig. 5b). Thus, Ald2p and Ald3p were clearly not involved in acetate production in this mutant. We assessed the ACDH activity of the ald6Δald4Δald5Δ and aldΔ mutants during exponential growth on YPD medium, in the presence of both NAD+ and NADP+. Specific NAD(P)+-ACDH activity was five times higher in strain CEN.PK [163-7 mU (mg protein)-1] than in strain V5 [29-9 mU (mg protein)-1], consistent with the higher levels of acetate production in the CEN.PK strain (Table 3). No ACDH activity was detected in the various mutants. Attempts to enhance the detection of the signal by increasing the volume of extract were unsuccessful. This suggests that another pathway exists for the synthesis of acetate.

DISCUSSION

Role of ALD genes in acetaldehyde metabolism

Most previous studies on ACDH have been based on analysis of the growth phenotype of deletion mutants on various substrates (Navarro-Avino et al., 1999; Meaden et al., 1997; Tessier et al., 1998; Wang et al., 1997, 1998). In this study, we analysed both growth and acetate production of single and multiple deletion mutants to obtain new insights concerning the function and the relative contribution of each isoform during anaerobic glucose fermentation. We found that Ald6p plays a key role in acetate production during fermentation, in agreement with earlier studies (Meaden et al., 1997; Remize et al., 2000). We have demonstrated that Ald5p is also involved in the formation of acetate from acetaldehyde produced during fermentation. This isoform has been shown to play a role in respiratory metabolism. An ald5Δ mutant exhibited defective mitochondrial electron transport and low cytochrome levels, suggesting that Ald5p is involved in haem regulation or biosynthesis (Kurita & Nishida, 1999). In contrast, a role for Ald5p in growth on glucose was excluded in a study by Wang et al. (1998) because the growth of an ald6Δald4Δ mutant was marginal on 2% glucose plates. We provide several lines of evidence in this study that Ald5p is indeed involved in acetate synthesis during glucose fermentation. First, ALD5 was strongly expressed in the exponential phase of growth in MS; second, the deletion of ALD5 significantly decreased acetate formation (by 22–26%) in the two strains studied; and third, the additional deletion of ALD5 slightly reduced acetate formation in the ald6Δ mutant and delayed growth in the ald6Δald4Δ mutant.

It has been suggested that both Ald6p and Ald4p could contribute to the formation of acetate during fermentation, based on the slow growth on glucose of mutant ald6Δald4Δ
(Wang et al., 1998). Results from expression studies and analysis of ald4 deletion mutants in V5 (MS and YPD medium) and CEN.PK (MS) support the idea that Ald4p is not involved in acetate formation. This is consistent with the fact that the K⁺-mitochondrial isofrm is strongly repressed by glucose (Llorente & Nunez de Castro, 1977). However, the role of Ald4p may depend both on strain genetic background and on medium composition since deletion of ALD4 slightly decreased the level of acetate formed in the CEN.PK strain when grown on YPD only. A possible explanation for the difference observed between MS 15 % glucose and YPD 5 % glucose medium may rely on a different level of glucose repression. It has been shown that the NAD⁺-linked activity found in cells grown on 0·5 % glucose could not be detected in cells grown on 10 % glucose (Llorente & Nunez de Castro, 1977), but the level of repression as a function of glucose concentration has not been investigated. To test this hypothesis, we examined the impact of the deletion of ALD4 on YPD 5, 10 and 15 % glucose. We found that deletion of ALD4 in strain CEN.PK did not significantly affect the level of acetate produced at concentrations higher than 5 % (data not shown), supporting the view that Ald4p might be totally repressed on MS but not on YPD. The different behaviour of the ald4 mutant in the CEN.PK and V5 strains might be due to differences in the regulation of the corresponding isoforms or to other genetic variations. Major differences have been reported in the phenotype of ald4Δ mutants; growth on ethanol was abolished or impaired, depending on genetic background (Tessier et al., 1998; Wang et al., 1998). These differences have been explained by a partial compensation of the cytosolic ACDH for the lack of ALD4 only when the activity of the cytosolic enzyme is sufficient (Boubekeur et al., 2001).

We showed during this work that Ald4p can compensate for the lack of Ald6p in yeast grown on glucose, and that this compensation requires the induction of ALD4 transcription. The mechanism underlying this induction remains to be elucidated. As K⁺-ACDH is subject to glucose repression, we cannot exclude that ALD4 is derepressed in ald6Δ mutants. This hypothesis is supported by the finding that a ald6Δ mutant exhibited an increase in K⁺-ACDH activity on 5 % glucose that was not observed on 0·5 % glucose (Tessier et al., 1999).

The roles played by the products of the closely related ALD2 and ALD3 genes in acetaldehyde metabolism are unclear. The lower growth rate on ethanol of the ald2Δald3Δ mutant suggested a possible role for these isoforms in ethanol oxidation (Navarro-Avino et al., 1999). Other studies have suggested that these isoforms may be involved in redox metabolism, particularly under conditions of osmotic stress (Navarro-Avino et al., 1999; Blomberg & Adler, 1989). Our results show that Ald2p and Ald3p are not required for acetate production during growth on glucose, even under wine fermentation conditions, in which yeast cells are exposed to osmotic stress due to the large amount of sugar present. Very recently, these two proteins, which are more distantly related to ACDH (Navarro-Avino et al., 1999; Meadon et al., 1997), were shown to have a specialized function in coenzyme A biosynthesis, converting 3-aminopropanal to β-alanine (White et al., 2003).

**Role of the mitochondrial PDH bypass during anaerobic glucose fermentation**

Overall, our data show that the cytosolic PDH bypass and a mitochondrial bypass may function simultaneously to produce acetate during fermentation (Fig. 6). Acetaldehyde produced from pyruvate is converted to acetate by Ald6p in the cytosol and by Ald5p in the mitochondria, and this results in the generation of NADPH reducing equivalents. Acetate formed in the mitochondria is then exported to the cytosol where it is converted to acetyl-CoA. When ALD6 is lost, acetate is formed by the mitochondrial enzymes.

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**Fig. 6.** Pathways for acetate production during fermentation. Acetate is mainly produced during glucose fermentation by the NADP⁺-dependent cytosolic Ald6p and mitochondrial Ald5p ACDH isoforms. In the absence of the cytosolic Ald6p isoform, acetate is produced by the mitochondrial route, by Ald5p and Ald4p, the latter of which is overproduced. Ald2p and Ald3p are not involved in acetate production during fermentation.
However, in this case overproduction of Ald4p is necessary to compensate efficiently for the loss of Ald6p.

Under the experimental conditions used in this study, traces of oxygen present at the start of the fermentation might increase the activity of the mitochondrial isoforms. However, this was not the case as the growth phenotype of V5 mutants was similar under strict anaerobic conditions (data not shown). Therefore, the mitochondrial pathway is operative under strict anaerobic conditions. A mitochondrial PDH bypass contributing to the oxidative metabolism of pyruvate has been described (Boubekeur et al., 1999). This pathway, which depends solely on the Ald4p isoform, can compensate for the absence of PDH in yeast grown aerobically on glucose or lactate, because both NADH and acetate are produced in the mitochondria. The mitochondrial bypass operating during growth on glucose, as reported in this study, cannot be involved in energy metabolism because the respiratory chain is inactive under anaerobic conditions, and because Ald5p uses NADP⁺ rather than NAD⁺ as a cofactor. Instead, the mitochondrial ACDH route might play a role in anaerobic redox metabolism, in addition to its function as a salvage pathway for acetyl-CoA. Biological membranes are impermeable to redox cofactors. The regeneration of these metabolites must therefore balance in each compartment. Ald6p plays an important role in the production of cytosolic NADPH. A zwf1Δald6Δ mutant was recently found to be unable to grow on glucose, demonstrating that Ald6p is the sole source of cytosolic NADPH other than the pentose-phosphate pathway (Grabowska & Chelstowska, 2003). Ald6p and Ald5p are located in the cytosol and in the mitochondria, respectively, and both these isoforms preferentially use NADP⁺ as a cofactor. The similar expression patterns of ALD6 and ALD5 during wine fermentation suggests that these two isoforms play a similar physiological role in S. cerevisiae. Only two reactions are known to generate NADPH in the mitochondria: the reaction catalysed by malic enzyme encoded by MAE1 and the reaction catalysed by the NADP⁺-dependent isocitrate dehydrogenase encoded by IDP1. The reaction catalysed by Ald5p may also play a role in the supply of NADPH in mitochondria.

In the ald6 mutants, only the mitochondrial route is operative. Since redox balance has to be achieved in individual compartments, the compensation of the mitochondrial enzymes for the loss of ALD6 would result in redox imbalance. A first possibility to account for the expected imbalance is a relocalization of the Ald4p isoform in the cytosol. However, protein fractionation experiments allowed us to discard this hypothesis (data not shown), suggesting that the demand for reducing equivalents is handled in a different manner.

**Alternative pathways for acetate production**

The aldΔ mutant, in which all five members of the ACDH family have been deleted, is viable. This mutant, and the ald6Δald4Δald5Δ mutant, both produced acetate, allowing acetyl-CoA production in the cytosol. The phenotype of a mutant devoid of PDC has demonstrated the absence of any other pathway for acetate production in the cytosol. Indeed, a pdcΔ mutant can only grow on minimal medium with glucose if the medium is supplemented with acetate (Pronk et al., 1996). A currently unknown pathway, normally inoperative on glucose, may therefore be deregulated to produce acetate in mutants devoid of ACDH activity. There are several possible ways in which acetate may be formed in the ald6Δald4Δald5Δ and aldΔ mutants. First, acetate may be produced by another dehydrogenase enzyme, capable of using acetaldehyde as a substrate. The ALD genes belong to the larger family of aldehyde dehydrogenases, which also includes Msc7, Uga2, Put2, Ymr110c, Ykr096w and Yil151c. However, we detected no residual ACDH activity in the ald6Δald4Δald5Δ and aldΔ mutants, suggesting that this hypothesis is incorrect. The conversion of acetaldehyde to acetate and ethanol is another possible source of acetate and such a conversion may be carried out by the dismutase activity which is a known secondary activity of alcohol dehydrogenases. Such activity has been described in various organisms, including humans, Drosophila melanogaster and Alcaligenes eutrophus (Svensson et al., 1999; Winberg & McKinley-McKee, 1998; Steinbüchel & Schlegel, 1984), but has not yet been reported in yeast. Alternatively, acetate may be formed by hydrolysis of mitochondrial acetyl-CoA. S. cerevisiae has an acetyl-CoA hydrolase, which is glucose-repressed and which has been shown recently to be localized in the mitochondria (Buu et al., 2003). Investigations are under way to examine these possibilities further.

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**REFERENCES**


