Specialization of Function Among Aldehyde Dehydrogenases: The *ALD2* and *ALD3* Genes Are Required for β-Alanine Biosynthesis in *Saccharomyces cerevisiae*

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ABSTRACT

The amino acid β-alanine is an intermediate in pantothenic acid (vitamin B_5) and coenzyme A (CoA) biosynthesis. In contrast to bacteria, yeast derive the β-alanine required for pantothenic acid production via polyamine metabolism, mediated by the four *SPE* genes and by the FAD-dependent amine oxidase encoded by *FMS1*. Because amine oxidases generally produce aldehyde derivatives of amine compounds, we propose that an additional aldehyde-dehydrogenase-mediated step is required to make β-alanine from the precursor aldehyde, 3-aminopropanal. This study presents evidence that the closely related aldehyde dehydrogenase genes *ALD2* and *ALD3* are required for pantothenic acid biosynthesis via conversion of 3-aminopropanal to β-alanine *in vivo*. While deletion of the nuclear gene encoding the unrelated mitochondrial Ald5p resulted in an enhanced requirement for pantothenic acid pathway metabolites, we found no evidence to indicate that the Ald5p functions directly in the conversion of 3-aminopropanal to β-alanine. Thus, in *Saccharomyces cerevisiae*, *ALD2* and *ALD3* are specialized for β-alanine biosynthesis and are consequently involved in the cellular biosynthesis of coenzyme A.

PANTOTHENIC acid (vitamin B_5) and β-alanine are intermediates in coenzyme A (CoA) biosynthesis. In bacteria, pantothenic acid is synthesized by the condensation of pantoate, an intermediate in valine biosynthesis, with β-alanine, produced by the decarboxylation of L-aspartate (Williamson and Brown 1979; Cronan 1980; Jackowski 1996). In yeast, the derivation of pantoate involves the same enzymatic steps as in bacteria, while β-alanine biosynthesis differs from that and is dependent upon polyamine degradation (mediated by the *SPE* genes) and upon the amine oxidase encoded by *FMS1* (White *et al.* 2001). Amine oxidases can degrade polyamines with the production of the aldehyde compound 3-aminopropanal (Hölttä 1977; Large 1992), implying that further oxidation of 3-aminopropanal by an aldehyde dehydrogenase would also be required for β-alanine biosynthesis in yeast.

The complete yeast genome encodes seven different members of the "nonspecific" aldehyde dehydrogenase family (Wang *et al.* 1998); see Table 1. *ALD2* and *ALD3* encode closely related cytosolic enzymes that are induced on ethanol media or in response to stress (Navarro-Aviño *et al.* 1999). *ALD4* encodes the major K^+-dependent mitochondrial enzyme (Jacobson and Bernofsky 1974; Tessier *et al.* 1998), and *ALD5* encodes a minor K^+-dependent mitochondrial enzyme that is induced on ethanol (Kurita and Nishida 1999). *ALD6* encodes a Mg^{2+}-activated cytosolic enzyme (Dickinson 1996; Meaden *et al.* 1997). *MSC7/YHR039c* encodes a protein with homology to aldehyde deydrogenases that affects meiotic sister-chromatid recombination (Thompson and Stahl 1999), and *YMR110c* is a hypothetical open reading frame that could code for an aldehyde-dehydrogenase-related protein.

Despite the multiple genes, only one physiological substrate, acetaldehyde, has been identified. Ald4p and Ald6p function in the production of acetate from acetaldehyde, a key intermediate during fermentation of sugars as well as during growth on ethanol, and are consequently important for acetyl-CoA production (Dickinson 1996; Meaden *et al.* 1997; Wang *et al.* 1998; Tessier *et al.* 1999; Remize *et al.* 2000). In contrast, Ald2p, Ald3p, and Ald5p do not contribute to the oxidation of acetaldehyde *in vivo* (Wang *et al.* 1998; Navarro-Aviño *et al.* 1999; Remize *et al.* 2000). The double-deletion mutant (*ald2*Δ *ald3*Δ) has been reported to grow slowly on ethanol, suggesting that Ald2/3p has a function under those conditions (Navarro-Aviño *et al.* 1999). An undefined role for Ald5p in heme biosynthesis has been prosposed, based on defective mitochondrial electron transport and the lack of cytochromes in the mitochondria of an *ald5*Δ deletion strain (Kurita and Nishida 1999). Consequently, it remains unclear what role these enzymes play in biosynthetic pathways or in the detoxification of exogenous aldehydes.

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TABLE 1

Aldehyde dehydrogenase genes of *S. cerevisiae*

ORF name[a]	Gene name	Subcellular localization	Function[b]
YMR170c	*ALD2*	Cytosolic	β-Alanine biosynthesis
YMR169c	*ALD3*	Cytosolic	β-Alanine biosynthesis
YOR374w	*ALD4*	Mitochondrial	Acetate biosynthesis
YER073w	*ALD5*	Mitochondrial	Mitochondrial function
YPL061w	*ALD6*	Cytosolic	Acetate biosynthesis
YHR039c[c]	MSC7	Unknown	Meiotic recombination
YMR110c[c]	—	Unknown	Unknown

ORF, open reading frame.

[a] Open reading frame designations used in the YPD (Hodges *et al.* 1999) and SGD (http://genome-www.stanford.edu/Saccharomyces/).

[b] Functions reported previously (see Introduction), except for β-alanine biosynthesis, which is the subject of this report.

[c] Putative aldehyde dehydrogenase genes.

In this study, we present evidence that *ALD2* and *ALD3* are specifically required for the conversion of 3-aminopropanal to β-alanine in the metabolic pathway leading to pantothenic acid and coenzyme A. Despite a significant degree of amino acid conservation, none of the other aldehyde dehydrogenase genes play a role in β-alanine production. These findings suggest that the "nonspecific" aldehyde dehydrogenases are functionally specialized to carry out different roles in cellular biosynthesis and therefore in fact have specific and differentiated biochemical functions.

MATERIALS AND METHODS

Yeast strains and gene deletions: The parental yeast haploid, BY4742, and its gene deletion strain derivatives from the Saccharomyces Deletion Project (SGD; Winzeler *et al.* 1999) were obtained from Research Genetics (Huntsville, AL): BY4742 (*MAT*α *his3 leu lys2 ura3*), BY4742-10753 (*ald2*Δ), BY4742-10752 and BY4742-16071 (*ald3*Δ), BY4742-11671 (*ald4*Δ), BY4742-10213 (*ald5*Δ), BY4742-12767 (*ald6*Δ), BY4742-11002 (*yhr039c*Δ), BY4742-16550 (*ymr110c*Δ), BY4742-10595 (*fms1*Δ), BY4742-13316 (*ecm31*Δ), and BY4742-12304 (*pan6*Δ = *yil145c*Δ). The corresponding homozygous diploid deletion strains were obtained from the same source. The identities of the *ald*Δ strains were confirmed by PCR amplification of genomic DNA using oligonucleotides designed for this purpose by the SGD (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html). The *ald2*Δ*ald3*Δ*::HIS3* double deletion was constructed by microhomologous recombination (Manivasakam *et al.* 1995) with a DNA construct made by PCR amplification. Oligonucleotide primers, DD5, 5′-tgctcaa gaatgttcatataaagggagcgaatccttaaaaggggatccggtgattgattg-3′, and DD3, 5′-taatatttcattctcttacgcttagcttacatggctgcgctggctgcaggtcga cggatc-3′ (Sigma-Genosys, The Woodlands, TX), were used to prime PCR amplification of the *HIS3* gene cassette from plasmid YDpH (Berben *et al.* 1991). This resulted in a DNA fragment containing the *HIS3* gene flanked by 40 bp of DNA homologous to DNA sequences outside the tandem repeated sequence of the *ALD2-ALD3* locus. His^+ transformants were then selected in the haploid strain BY4742, and deletion of the *ALD2-ALD3* locus was confirmed by PCR amplification of genomic DNA using two pairs of primers: 169seq, 5′-ttgtgat cacctgctctctg-3′, with 170seq, 5′-cttgtcgacactcactgatc-3′, which amplifies both wild-type and deleted loci, and HIS3, 5′-ggtggag ggaacatcgttgg-3′, with 170seq, which produces a PCR product only in the *ald2*Δ*ald3*Δ*::HIS3* deletion strain. The *ald2*Δ *ald5*Δ*::URA3* and *ald3*Δ*ald5*Δ*::URA3* double-deletion strains were constructed in a similar fashion. Oligonucleotide primers A55, 5′-aacttcttcacaacattaacaaaaagccaaagaagaagaaggggatccgg tgattgattg-3′ and A53, 5′-tctataatgtttatcatacatacctttcaatgagcagtc aatggctgcaggtcgacggatc-3′, were used to prime PCR amplification from plasmid YDpU (Berben *et al.* 1991), resulting in a DNA fragment containing the *URA3* gene flanked by 40 bp of DNA homologous to the DNA sequence just outside of the *ALD5* open reading frame. Ura^+ transformants were selected in strains BY4742-10753 (*ald2*Δ) and BY4742-16071 (*ald3*Δ), and deletion of the *ALD5* gene was confirmed by PCR amplification of genomic DNA using the primer pair A5validFOR, 5′-cgatgagaatggcttcaaag-3′, with URA3, 5′-cctttgttacttcttccgcc-3′, which produces a 1.3-kb PCR product only at an *ald5*Δ*::URA3* locus.

Yeast plasmids: Genomic DNA fragments carrying the *ALD2*, *ALD3*, and *ALD5* genes with flanking sequences were individually subcloned into the *Bam*HI site of the *CEN-LEU2* yeast shuttle vector YCplac111 (Gietz and Sugino 1988) or pRS315 (Sikorski and Hieter 1989). The genomic DNA fragments were made by PCR using total yeast genomic DNA as the reaction template and gene-specific oligonucleotides. For *ALD2*, a 2298-bp subclone was obtained using oligonucleotides 5′-ccctttggatccgctacctcttaatgtgtcac-3′ and 5′-ccctttggatccaagat ctacgtaatggtggg-3′. For *ALD3*, a 2227-bp subclone was obtained using the oligonucleotides 5′-ccctttggatcccatatgacgtctgttcttcc-3′ and 5′-ccctttggatccacattcggagtcctgtcctc-3′. For *ALD5*, a 2471-bp subclone was obtained using the oligonucleotides 5′-ccctttgg atccctcttgtggctatgtaagcc-3′ and 5′-ccctttggatccctctggcacttgtatc tacc-3′.

The yeast expression vector YEp195AC and the *ADH1-FMS1* derivative for overexpression of the *FMS1* gene are 2μ-based vectors containing the *URA3* selectable marker gene, as previously described (White *et al.* 2001). Plasmids were introduced into yeast cells using lithium acetate (Gietz and Schiestl 1995).

Media and growth conditions: Media lacking pantothenic acid and halo assays were as previously described (White *et al.* 2001). However, for experiments with *ald5*Δ strains, the addition of complete amino acid supplements was found to enhance the pantothenate auxotrophy. Pantothenic acid, β-alanine, or spermine (Sigma, St. Louis) were added at the concentrations indicated in the text. 3-Aminopropanal (3-aminoproprionaldehyde) was made by hydrolysis of 3-aminoproprionaldehyde diacetal (Acros, Fairlawn, NJ, no. 269850250); a 10 mm solution of the diacetal was incubated in 1 m HCl at room temperature for 2 hr and then neutralized with 5 m KOH before being added to growth media. YPD media was 2% glucose, 2% bactopeptone, and 1% yeast extract (Difco). Anaerobic growth conditions were obtained using a GasPak 100 jar (Becton Dickinson no. 260626) with GasPak Plus envelopes and anaerobic indicators (Becton Dickinson nos. 271040 and 271051). Solid NaCl, KCl, or $CaCl_2$ (Sigma) was added directly to liquid medium to final concentrations indicated in the text and figure legends. The liquid growth curve was established by standard methods, using 96-well flat-bottom

polystyrene assay plates and a Spectramax 384 Plus (Molecular Devices, Sunnyvale, CA).

RESULTS

***ALD2* and *ALD3* mediate the conversion of 3-aminopropanal to β-alanine *in vivo*:** While testing each of the *ald* gene deletion strains for pantothenic acid auxotrophy by the traditional replica-plating technique, we found that only the double deletion *ald2*Δ*ald3*Δ exhibited complete pantothenic acid auxotrophy. This auxotrophy can be complemented by introduction of either the *ALD2* or the *ALD3* plasmids (see MATERIALS AND METHODS), confirming that the double deletion is the cause of the phenotype. Strain BY4742, its single gene deletion derivatives *fms1*Δ, *ecm31*Δ, and *pan6*Δ, and the double-deletion strain *ald2*Δ*ald3*Δ were replica plated onto media lacking pantothenic acid or supplemented with 3-aminopropanal, β-alanine, or pantothenic acid (Figure 1A). All of these strains could grow when pantothenic acid was added to the media. However, the *ald2*Δ *ald3*Δ strain could grow with β-alanine instead of with pantothenic acid, and the *fms1*Δ strain could grow either on 3-aminopropanal or on β-alanine instead of on pantothenic acid. This indicates that *ALD2* and *ALD3* function downstream of *FMS1* in the β-alanine and pantothenic acid biosynthetic pathway and are specifically required for the utilization of 3-aminopropanal. This is consistent with the model of 3-aminopropanal conversion via β-alanine to pantothenic acid, requiring the genes *FMS1*, *ALD2* or *ALD3*, *ECM31*, and *PAN6*, respectively (Figure 1B).

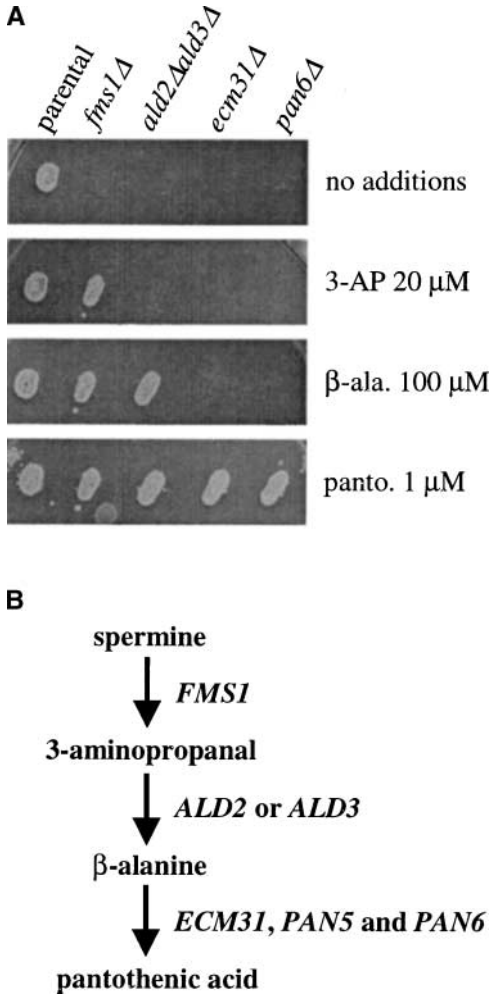

FIGURE 1.—Auxotrophic phenotypes caused by deletion of genes required for pantothenic acid biosynthesis. (A) Parental strain BY4742 and its deletion derivatives *fms1*Δ, *ald2*Δ*ald3*Δ, *ecm31*Δ, and *pan6*Δ were patched onto YPD agar media and incubated at 30° overnight to allow growth. The YPD plate was then replica plated onto media lacking pantothenic acid ("no additions") and supplemented with 20 μM 3-aminopropanal (3-AP), 100 μM β-alanine (β-ala.), or 1 μM pantothenic acid (panto.), as indicated. The replica plates were incubated for 2 days at 30°. (B) The order of the biochemical intermediates, and the order of function of the genes required, inferred from the replica-plating experiment in A.

Because the single deletions *ald2*Δ and *ald3*Δ, as well as the single deletions of the remaining aldehyde dehydrogenase genes, did not appear to require exogenous β-alanine or pantothenic acid for growth using the replica plate method (data not shown), we sought a more sensitive plating technique to assess pantothenic acid pathway metabolite requirements. We found that if an inoculum of fewer cells is used (by "spotting" a dilute liquid suspension of cells onto agar medium), a more sensitive assay is produced and partial auxotrophies can be detected. Therefore, five of the single deletion strains, *ald2*Δ–*ald6*Δ, the double deletion *ald2*Δ*ald3*Δ, and the parental strain BY4742 were spotted onto medium lacking pantothenic acid or onto medium supplemented with spermine, 3-aminopropanal, β-alanine, or pantothenic acid (Figure 2). In contrast to the replica-plating result, the *ald2*Δ single mutant did not grow in the absence of β-alanine or pantothenic acid after 3 days of incubation. This auxotrophy was completely reversed by introduction of a plasmid carrying the *ALD2* locus (see MATERIALS AND METHODS, data not shown). Although this is the same phenotype as the *ald2*Δ*ald3*Δ strain in the replica plate assay, it suggests that the single mutant *ald2*Δ is less deficient than the *ald2*Δ*ald3*Δ mutant in pantothenic acid biosynthesis. In this sensitive spotting assay, increased inoculum size or an extended incubation time (*e.g.*, to 7 days) allowed for detectable growth of the *ald2*Δ single mutant but not of the *ald2*Δ *ald3*Δ double mutant (not shown).

***ALD5* does not play a role in pantothenic acid metabolism:** In the spotting assay, a second deletion mutant, *ald5*Δ, also did not grow on media lacking β-alanine or pantothenic acid (Figure 2A). The *ald5*Δ phenotype could be rescued by the introduction of plasmids carrying the *ALD5* locus (see MATERIALS AND METHODS), and, in addition, the homozygous diploid strain *ald5*Δ/*ald5*Δ behaved in an identical manner (not shown), indicating that the *ald5*Δ deletion was the cause of the

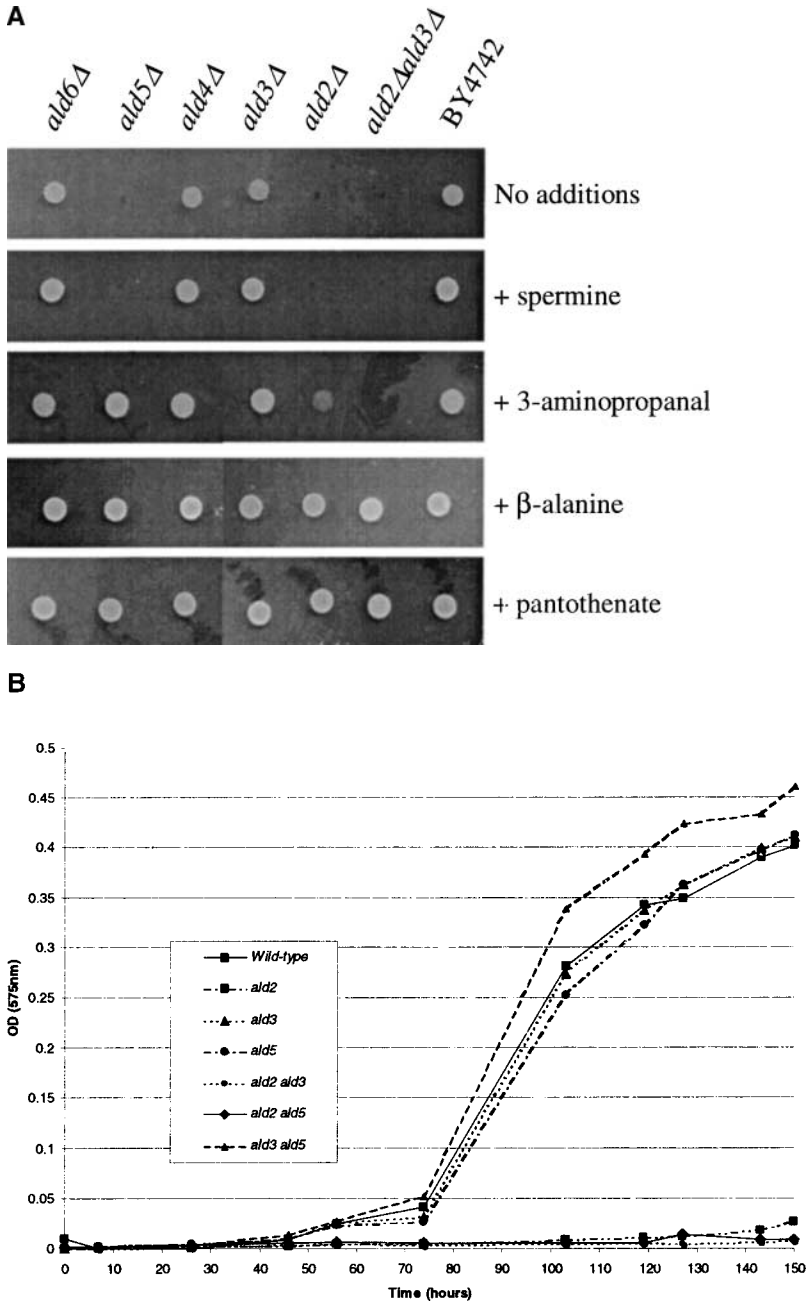

FIGURE 2.—Auxotrophic phenotypes caused by deletion of the aldehyde dehydrogenase genes. (A) Parental strain BY4742 and its deletion derivatives *ald2*Δ, *ald3*Δ, *ald4*Δ, *ald5*Δ, and *ald6*Δ, and the double deletion *ald2*Δ*ald3*Δ were grown overnight on medium lacking pantothenic acid (to exhaust internal pools of pantothenic acid metabolites). Cells were then harvested and washed in distilled water by centrifugation, and 5-μl droplets containing *ca.* 2000 cells were inoculated onto agar medium lacking pantothenic acid ("No additions") or supplemented with 1 μM pantothenate, 100 μM spermine, 100 μM 3-aminopropanal, or 200 μM β-alanine, as indicated. Incubation was for 3 days at 30°. (B) Parental strain BY4742, deletion derivatives *ald2*Δ, *ald3*Δ, and *ald5*Δ, and the double deletions *ald2*Δ*ald3*Δ, *ald2*Δ*ald5*Δ, and *ald3*Δ *ald5*Δ were grown for 3 days on medium lacking pantothenic acid. Cells were harvested and washed in distilled water by centrifugation, and ~2000 cells were inoculated into 50 ml of liquid media lacking pantothenic acid. Cultures were incubated at 30° with shaking at 200 rpm, and optical density measurements (575 nm) were taken at various intervals out to 150 hr.

phenotype in this strain. In contrast to the *ald2*Δ strain, 3-aminopropanal supported strong growth of the *ald5*Δ strain (Figure 2A), indicating that the *ald5*Δ mutant was not defective for the conversion of 3-aminopropanal to β-alanine. Because it grew on 3-aminopropanal but not on spermine, the requirements of the *ald5*Δ mutant for pantothenic acid pathway metabolites resembled those of *fms1*Δ. However, we observed several phenotypic differences (not shown): first, the *ald5*Δ strain required more exogenous β-alanine than the *fms1*Δ mutant did for strong growth in the spotting assay. Second, in the absence of pantothenic acid, increased inoculum size (*e.g.*, replica plating) allowed for growth of the *ald5*Δ but not of the *fms1*Δ strain. Third, the addition of multiple amino acid supplements enhanced the β-alanine auxotrophy of the *ald5*Δ mutant, consistent with an indirect role for the Ald5p enzyme in pantothenic acid biosynthesis.

Although these results indicate that Ald5p does not function directly in 3-aminopropanal conversion to β-alanine, we sought a more sensitive and definitive measure of the phenotype of *ald5*Δ in pantothenic acid metabolism as it relates to growth and viability, particularly in the context of *ald2*Δ and *ald3*Δ mutations. Therefore, we quantified the growth capabilities of the parental strain BY4742, double mutants *ald2*Δ*ald5*Δ, *ald3*Δ*ald5*Δ, and *ald2*Δ*ald3*Δ, as well as the single mutants *ald2*Δ, *ald3*Δ, and *ald5*Δ, in the absence of exogenous pantothenic

acid. Both total growth and the apparent log-phase growth rate of the parental, *ald3*Δ, *ald5*Δ, and *ald3*Δ *ald5*Δ strains were approximately equivalent, while growth of the *ald2*Δ, *ald2*Δ*ald3*Δ, and *ald2*Δ*ald5*Δ strains remained at or near zero (Figure 2B). The fact that the *ald5*Δ has no discernible effect on growth in this assay does not contradict the spotting assay result, because the terminal incubation time was much shorter in the spotting assay than in the liquid growth assay. In fact, the *ald5*Δ strain did yield visible growth in the spotting assay after 7 days of incubation (not shown). Taken together, these results clearly indicate that *ALD5* does not encode an aldehyde dehydrogenase required for the conversion of 3-aminopropanal to β-alanine and that Ald5p is unlikely to be involved either directly or indirectly in pantothenic acid metabolism.

***ALD2* plays the predominant role in pantothenic acid production:** On glucose medium, endogenous *FMS1* expression is rate limiting for both growth rate and pantothenic acid production. When *FMS1* is overexpressed using the *ADH-FMS1* allele on a plasmid, growth rate is accelerated and excess pantothenic acid is excreted into the medium. This excretion can be detected using a bioassay involving growth of a pantothenic acid auxotroph, such as the *ecm31*Δ mutant (White *et al.* 2001). We therefore compared the phenotypes of *ald2*Δ, *ald3*Δ, and *ald5*Δ mutants further by testing the ability of these deletion strains to excrete pantothenic acid. Strains BY4742, *ald2*Δ, *ald3*Δ, and *ald5*Δ harboring the *ADH-FMS1* plasmid were spotted onto a "lawn" of *ecm31*Δ cells, which require pantothenic acid for growth. After incubation, halos of growth formed around all spots except around the *ald2*Δ mutant. Again, this suggests that *ALD2* is directly involved in pantothenic acid production and is responsible for the majority of the conversion from 3-aminopropanal to β-alanine (Figure 3A). Halos of growth did not occur around any of the strains harboring an empty vector control (not shown).

Another way to analyze the function of genes in the pantothenic acid pathway is to test the ability of the *ADH1-FMS1* overexpression allele to rescue growth on medium lacking pantothenic acid. Strains lacking enzymes in the same pathway as *FMS1* will be unable to grow, while strains lacking enzymes in unrelated pathways will grow. The deletion strains, *ald2*Δ, *ald5*Δ, and *ald2*Δ*ald3*Δ, were transformed with the *ADH1-FMS1*, or empty vector YEp195AC, and tested for growth on medium lacking pantothenic acid (Figure 3B). The *ald2*Δ *ald3*Δ strain completely blocked the *ADH1-FMS1*-dependent stimulation of growth in the absence of pantothenic acid. Likewise, the *ald2*Δ strain was defective, although slow growth occurred with extended incubation time. Unlike the phenotype observed in the *ald2*Δ and *ald2*Δ*ald3*Δ mutants, the *ADH1-FMS1* allele rescued growth in the *ald5*Δ mutant in this assay, again indicating that *ALD5* does not affect pantothenic acid biosynthesis. Taken together, these lines of evidence indicated

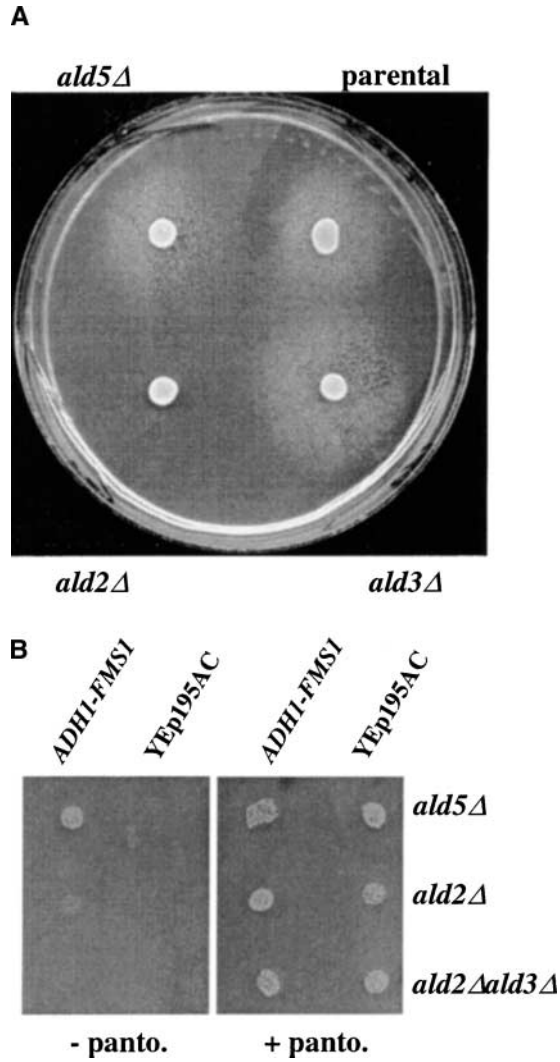

Figure 3.—Pantothenic acid overproduction and excretion are dependent upon *ALD2*, but not upon *ALD3* or *ALD5*. (A) Pantothenic acid excretion requires *ALD2* activity. Parental strain BY4742 and its deletion derivatives *ald2*Δ, *ald3*Δ, and *ald5*Δ, harboring the *ADH1-FMS1* overexpression allele, were spotted (~1×10^7 cells/spot) onto a lawn of the *ecm31*Δ deletion strain on media lacking pantothenic acid. Incubation was for 2 days at 30°, after which time halos of growing lawn cells could be seen surrounding yeast spots that were excreting pantothenic acid. (B) Rescue of *ald*Δ strains by *FMS1* overexpression. Deletion strains *ald5*Δ, *ald2*Δ, and *ald2*Δ*ald3*Δ, harboring the *ADH1-FMS1* overexpression plasmid or the control vector YEp195AC, were spotted onto media containing pantothenic acid (+ panto.) or lacking pantothenic acid (− panto.), as indicated. Incubation was for 2 days at 30°.

that *FMS1* and *ALD2/ALD3* genes function together in the same pathway to mediate β-alanine and pantothenic acid biosynthesis.

***ALD3* compensates for the loss of *ALD2* under conditions of osmotic stress:** The pantothenic acid excretion bioassay results, combined with the pantothenic acid auxotrophy profiles of the single mutants *ald2*Δ or *ald3*Δ and the double mutant *ald2*Δ*ald3*Δ, suggested that while both *ALD2* and *ALD3* function in the conversion of

3-aminopropanal to β-alanine, the majority of this activity was dependent upon *ALD2*. Because previous evidence has indicated that the transcription of *ALD2* and *ALD3* is modulated by osmotic stress (Miralles and Serrano 1995; Navarro-Aviño *et al.* 1999), we reasoned that the growth defect of the *ald2*Δ strain on medium lacking exogenous β-alanine might be remediated under salt-induced osmostress, where *ALD3* is upregulated. Single mutants *ald2*Δ and *ald3*Δ, the double mutant *ald2*Δ*ald3*Δ, and the β-alanine auxotroph *fms1*Δ were therefore streaked onto medium lacking β-alanine, medium lacking β-alanine but containing 0.5 m NaCl, or medium supplemented with β-alanine (Figure 4). On medium lacking β-alanine, sectors containing *ald2*Δ cells did not have any appreciable growth. However, growth of the *ald2*Δ strain was evident on medium lacking β-alanine that contained 0.5 m NaCl. The *ald3*Δ strain grew under all conditions, while both *ald2*Δ*ald3*Δ and *fms1*Δ strains grew only when exogenous β-alanine was added to the medium. In the absence of exogenous β-alanine, the effect of 0.3 m KCl on growth of the *ald2*Δ strain was less pronounced than that of 0.5 m NaCl, and 0.5 m $CaCl_2$ had no effect on the growth of any of the strains (not shown). The observation that the double mutant *ald2*Δ*ald3*Δ remained auxotrophic for β-alanine in the presence of 0.5 m NaCl or 0.3 m KCl suggested that *ALD3*, and not one of the other aldehyde dehydrogenase genes, was responsible for growth of the *ald2*Δ strain under conditions of Na^+ or K^+ stress.

Molecular oxygen is required for pantothenic acid biosynthesis: Because amine oxidases utilize molecular oxygen (O_2) as a cosubstrate (in addition to amine compounds), O_2 should be required for pantothenic acid biosynthesis. To test this, strain BY4742 was streaked onto medium containing or lacking pantothenic acid and incubated under conditions of limited O_2 in an anaerobic jar. Control cultures were incubated at the same time on identical medium under aerobic conditions. After incubation for 3 days, growth was inhibited when pantothenic acid and O_2 were both absent, but growth took place when either pantothenic acid or O_2 was present (Figure 5). On a glucose medium, O_2 is required for a number of biosynthetic reactions, including sterol and unsaturated fatty acid synthesis. Presumably, these processes are sustained by the low level of O_2 remaining in the anaerobic jar, whereas the additional metabolic burden of pantothenic acid biosynthesis requires a higher concentration of O_2.

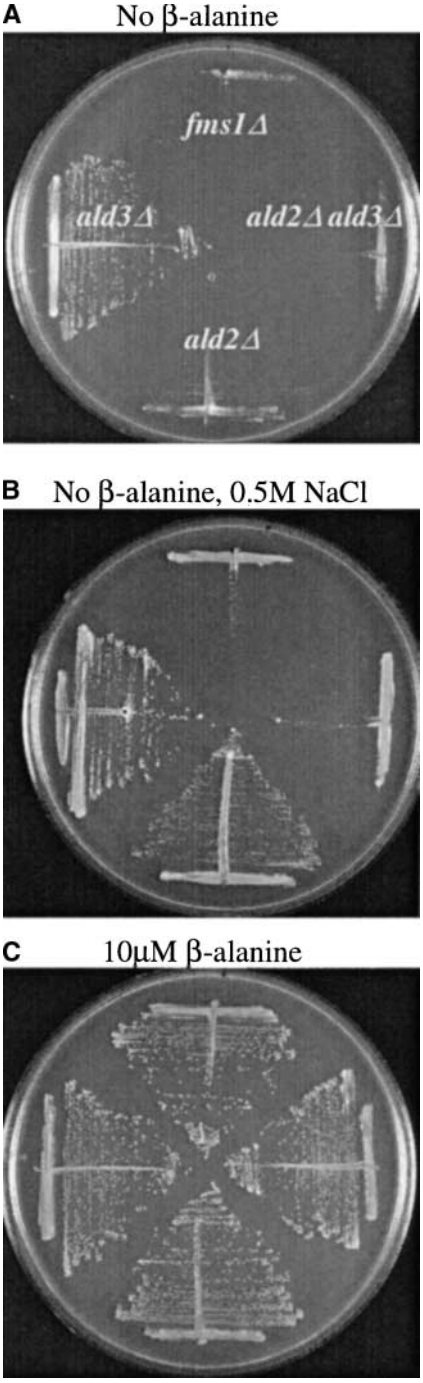

Figure 4.—NaCl-induced osmotic stress causes *ALD3*-dependent remediation of the β-alanine auxotrophy of *ald2*Δ. Yeast deletion strains *fms1*Δ, *ald2*Δ*ald3*Δ, *ald2*Δ, and *ald3*Δ (as indicated) were grown overnight in liquid medium that lacked pantothenic acid or β-alanine (to exhaust internal pools of the vitamin), then streaked onto (A) the same medium, (B) the same medium containing 0.5 m NaCl, and (C) the same medium containing 10 μm β-alanine, and then incubated for 3 days at 30°.

DISCUSSION

Aldehyde dehydrogenases are required for pantothenic acid biosynthesis in yeast: In yeast, the β-alanine required for pantothenic acid biosynthesis is derived from the oxidation of the polyamine spermine, involving the amine oxidase encoded by *FMS1* (White *et al.* 2001). In other organisms, this oxidation of polyamines to carboxylic acids occurs in two steps: First, in the presence of O_2, amine oxidases convert the polyamines to aldehydes, and second, aldehyde dehydrogenases

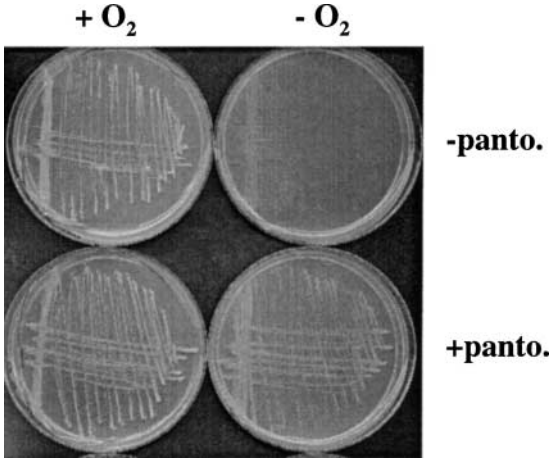

FIGURE 5.—O_2 is required for the growth of yeast in the absence of pantothenic acid. Yeast strain BY4742 was streaked onto medium containing pantothenic acid (+panto.) or lacking pantothenic acid (−panto.), as indicated, and incubated anaerobically ($-O_2$) or in the presence of air ($+O_2$), as indicated. Incubation was for 3 days at 37°.

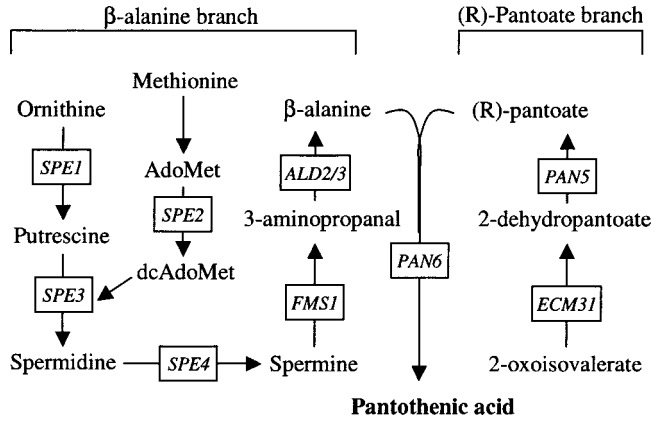

FIGURE 6.—The pantothenic acid pathway of yeast. The β-alanine branch involves degradation of polyamines, mediated by the *SPE* genes, the aldehyde dehydrogenase genes *ALD2* or *ALD3*, and the amine oxidase encoded by *FMS1*. The pantoate branch, involving *ECM31*, *PAN5*, and *PAN6*, is substantially the same as that found in bacteria. AdoMet, S-adenosyl methionine; dcAdoMet, decarboxy-S-adenosyl methionine.

convert the aldehydes to carboxylic acids (LARGE 1992). On the basis of this hypothesis of a two-step chemical conversion, we tested three predictions: first, that yeast can utilize 3-aminopropanal for pantothenic acid biosynthesis; second, that aldehyde dehydrogenase(s) is required to convert 3-aminopropanal to β-alanine; and third, that O_2 is required for pantothenic acid biosynthesis. We found that yeast was indeed able to utilize the aldehyde compound 3-aminopropanal for pantothenic acid production and that the aldehyde dehydrogenase genes *ALD2* and *ALD3* were required for the conversion of 3-aminopropanal to β-alanine *in vivo*. Finally, wild-type yeast had an increased requirement for O_2 during growth in the absence of pantothenic acid. Confirmation that *FMS1* functions in the same pathway as *ALD2* or *ALD3* came from pantothenic acid excretion experiments and complementation analysis, in which it was found that *FMS1* is functionally dependent on *ALD2* or *ALD3*. Thus, the amine oxidase Fms1p is required to make 3-aminopropanal from spermine, and the aldehyde dehydrogenases, Ald2p or Ald3p, are required to make β-alanine from 3-aminopropanal (Figure 6), consistent with the biochemistry of polyamine degradation in other organisms (LARGE 1992).

The role of aldehyde dehydrogenases in pantothenic acid biosynthesis is specific to *ALD2* and *ALD3*: *Saccharomyces cerevisiae* has seven known or putative "nonspecific" aldehyde dehydrogenases (WANG *et al.* 1998), and biochemical functions have been defined previously only for *ALD4* and *ALD6*, both of which convert acetaldehyde to acetate (see Introduction). *ALD6*, like *ALD2* and *ALD3*, encodes a cytosolic enzyme (MEADEN *et al.* 1997), but nevertheless we found it played no role in pantothenic acid biosynthesis. Thus, these three cytosolic aldehyde dehydrogenases are functionally specialized, most likely because they recognize different aldehyde compounds as substrates. On the other hand, the inability of the mitochondrial enzymes, Ald4p and Ald5p, to participate in pantothenic acid biosynthesis may be the result of not only substrate specificity (the Ald4p substrate is acetaldehyde), but also the subcellular location of the substrate in question; 3-aminopropanal is most likely produced and consumed in the cytosol, based on the localization of the enzymes involved.

The high degree of amino acid sequence identity between Ald2p and Ald3p, their genomic organization (encoded by 1518-bp tandem reading frames with 91% nucleotide homology, separated by a 690-bp intergenic region on chromosome XIII), and the stress response elements in their cognate promoters (NAVARRO-AVIÑO *et al.* 1999) suggest that the *ALD2-ALD3* locus may have arisen through a fairly recent gene duplication event. However, despite their 91% amino acid sequence identity, we cannot rule out the possibility that a degree of functional specialization may also exist between *ALD2* and *ALD3*, which is suggested by the finding that the *ald2*Δ strain was partially defective for pantothenic acid biosynthesis whereas *ald3*Δ was not defective in any of our assays. In other words, the participation of Ald3p in β-alanine production became evident only in the context of the *ald2*Δ background. The most straightforward explanation for this is that Ald2p is responsible for the majority of β-alanine production necessary for making pantothenic acid and that the phenotypic differences might simply be a reflection of different kinetic parameters with respect to conversion of 3-aminopropanal to β-alanine or of different protein expression levels within the cell. This was supported by our observation that the growth defect of the single mutant *ald2*Δ (but not the double mutant *ald2*Δ*ald3*Δ) on medium lacking

β-alanine was remediated under Na^+- or K^+-induced osmotic stress conditions. Since transcription of *ALD3* is known to be upregulated by osmostress (Navarro-Aviño *et al.* 1999), this suggested that increased *ALD3* transcription (in the presence of high levels of NaCl or KCl) provided sufficient converting activity of 3-aminopropanal to β-alanine to compensate for the absence of Ald2p, allowing the *ald2*Δ strain to grow in the absence of exogenous β-alanine. Additionally, the fact that deletion of both of these genes was required for complete β-alanine and pantothenic acid auxotrophy further indicated that Ald2p and Ald3p are both capable of converting 3-aminopropanal to β-alanine *in vivo*. It is also possible that *ALD2* and *ALD3* might have additional functions, as suggested by their role during growth on ethanol and by their regulated gene expression (Navarro-Aviño *et al.* 1999).

Although the *ald5*Δ mutant exhibited an enhanced requirement for pantothenic acid pathway metabolites in the spotting assay, several lines of existing and new evidence indicate that the Ald5p protein does not play an essential role in pantothenic acid biosynthesis. First, the mitochondrial localization of Ald5p is inconsistent with a role in cytosolic metabolism. The observation that *ald5*Δ strains are defective for mitochondrial electron transport and cytochrome biogenesis (Kurita and Nishida 1999) supports that proposition. Second, the amino acid identity of Ald5p with either Ald2p or Ald3p is much lower than that between Ald2p and Ald3p, which is consistent with the hypothesis of aldehyde dehydrogenase functional specialization. Third, we have shown that the *ald5*Δ mutant does not directly affect pantothenic acid biosynthesis *per se* in either the *ADH1-FMS1* allele complementation assay or the pantothenic acid excretion bioassay. Fourth, in liquid medium, the *ald5*Δ strain grew at a similar rate to the parental strain in the absence of exogenous pantothenic acid. Thus, the effect of *ald5*Δ on the pantothenic acid pathway is most likely of an indirect nature. Possible mechanisms include an increased cellular requirement for coenzyme A, a decreased Fms1p activity, and a decreased transport of spermine to the Fms1p enzyme. In all cases, overexpression of *FMS1* using the *ADH1-FMS1* allele would be expected to compensate for the partial pantothenate auxotrophy of *ald5*Δ either by increasing Fms1p activity or by putting Fms1p in parts of the cell it does not normally occupy. A full explanation will require a better understanding of the cellular role of *ALD5*.

In conclusion, the "nonspecific" aldehyde dehydrogenases do in fact have specialized functions in normal cellular metabolism. In the case of Ald2p and Ald3p, the function is in coenzyme A biosynthesis. This does not rule out possible "nonspecific" roles of these enzymes in protecting the cell from the occurrence of toxic aldehyde compounds, but does suggest that a number of different aldehyde dehydrogenase enzymes may be specialized for metabolic functions that occur during normal cellular growth and development.

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