

Aldehyde dehydrogenase is essential for both adult and larval ethanol resistance in *Drosophila melanogaster*

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Summary

The enzyme aldehyde dehydrogenase (ALDH) is essential for ethanol metabolism in mammals, converting the highly toxic intermediate acetaldehyde to acetate. The role of ALDH in *Drosophila* has been debated, with some authors arguing that, at least in larvae, acetaldehyde detoxification is carried out mainly by alcohol dehydrogenase (ADH), the enzyme responsible for converting ethanol to acetaldehyde. Here, we report the creation and characterization of four null mutants of *Aldh*, the putative structural locus for ALDH. *Aldh* null larvae and adults are poisoned by ethanol concentrations easily tolerated by wild-types; their ethanol sensitivity is in fact comparable to that of *Adh* nulls. The results refute the view that ALDH plays only a minor role in ethanol detoxification in larvae, and suggest that *Aldh* and *Adh* may be equally important players in the evolution of ethanol resistance in fruit-breeding *Drosophila*.

1. Introduction

Many *Drosophila* species breed in fermenting fruit, where ethanol can reach concentrations of several per cent (McKenzie & McKechnie, 1979; Gibson *et al.*, 1981). Ethanol has evidently been an important selective agent in the evolution of *Drosophila*, because fruit-breeding species are considerably more resistant to ethanol toxicity than species which breed in non-sweet vegetation such as mushrooms and decaying leaves (Merçot *et al.*, 1994). The model species *D. melanogaster* is one of the most ethanol-resistant *Drosophila*, with populations from temperate regions in particular being able to tolerate (David & Bocquet, 1975; Merçot *et al.*, 1994), and even use as a resource (Parsons *et al.*, 1979), concentrations of ethanol that would be lethal to most other fruit-breeding *Drosophila*.

Work on the genetic basis of ethanol resistance in *D. melanogaster* has focused almost exclusively on a single gene, *Alcohol dehydrogenase* (reviewed in Geer *et al.*, 1993; Heinstra, 1993). The ADH enzyme, which catalyses the oxidation of ethanol to acetaldehyde, is clearly essential for ethanol utilization and resistance.

Adh null flies are notoriously ethanol-sensitive (e.g., David *et al.*, 1976; Bijlsma & Bijlsma-Meeles, 1991), and ADH activity correlates with ethanol resistance among species (Merçot *et al.*, 1994). Moreover, within *D. melanogaster*, strains with the more enzymatically active *Fast* electromorph tend to have higher ethanol resistance than those with the *Slow* electromorph (reviewed in Heinstra, 1993). Nonetheless, *Adh* is clearly not the only gene responsible for variation in ethanol resistance within and among *Drosophila* species (Geer *et al.*, 1993; Chakir *et al.*, 1996; Fry *et al.*, 2004).

Another potentially important enzyme in the metabolism of ethanol is aldehyde dehydrogenase (ALDH; EC 1.2.1.3), which catalyses the oxidation of acetaldehyde to acetate. ALDH is believed to be the main enzyme responsible for the oxidation of acetaldehyde in mammals (Weiner, 1979). In humans, an inherited deficiency in the mitochondrial ALDH isozyme, ALDH2, causes a syndrome known as acute alcohol sensitivity (Impraim *et al.*, 1982; Yoshida *et al.*, 1984; Peng *et al.*, 1999). After ingesting small amounts of ethanol, affected individuals, who are mostly of east Asian descent, experience a variety of unpleasant symptoms caused by the accumulation of acetaldehyde, which is considerably more toxic than ethanol.

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While it has been clear for some time that *Drosophila* contain an active ALDH (Liétaert *et al.*, 1985; Garcin *et al.*, 1986; Leal & Barbancho, 1992), ALDH was initially hypothesized to be less important for ethanol metabolism in *Drosophila* than in mammals (Geer *et al.*, 1985; Heinstra *et al.*, 1989). This claim followed the discovery that *Drosophila* ADH is capable of oxidizing acetaldehyde to acetate by itself (Heinstra *et al.*, 1983; Eisses *et al.*, 1985; Geer *et al.*, 1985; but see Henehan *et al.*, 1995, for evidence that this ability may be more limited than concluded by the earlier studies). In contrast, available evidence at the time indicated that mammalian ADHs had at best limited ability to oxidize acetaldehyde (reviewed in Fry *et al.*, 2004). In apparent support of the view that ADH is the main enzyme responsible for acetaldehyde oxidation, pre-feeding an ALDH inhibitor caused only a small reduction (12–19%) in the flux of ethanol into lipid in larvae on a 1% ethanol diet (Heinstra *et al.*, 1989; Heinstra & Geer, 1991). In contrast, inhibiting ALDH in adults reduced survivorship in the presence of 5% ethanol from near 100% to 0 (Leal & Barbancho, 1992). These results led to the view that ALDH is important for ethanol catabolism in *Drosophila* adults but not larvae (e.g. Leal & Barbancho, 1992). Nonetheless, the degree of enzyme inhibition *in vivo* was not measured in the larval studies, and larval breeding sites often contain considerably higher concentrations of ethanol than was used in the studies. Therefore the studies do not rule out an important role for ALDH in ethanol resistance in larvae.

Null mutants of the ALDH structural locus would permit more definitive assessment of the role of ALDH in ethanol catabolism and resistance. The *D. melanogaster* genome contains a gene (*Aldh*, formerly CG3752) with 70% amino acid identity to human ALDH2, as well as several other more distantly related members of the aldehyde dehydrogenase family (Sophos & Vasiliou, 2003). Using deletion stocks, we tentatively identified *Aldh* as the gene responsible for most of the acetaldehyde dehydrogenase activity in flies (Fry *et al.*, 2004). Here, we describe the creation and characterization of a set of *Aldh* null mutants. The results show that *Aldh* is essential for ethanol resistance in both adults and larvae. Indeed, *Aldh* nulls are comparable in ethanol sensitivity to *Adh* nulls.

2. Materials and Methods

(i) Rearing conditions

Flies were maintained in shell vials on standard cornmeal–molasses–brewer's yeast–agar medium, at 25 °C under continuous light. Adults were handled under light CO₂ anaesthesia.

(ii) P element mobilization

This work made use of the following stocks (Lindsley & Zimm, 1992; Flybase Consortium, 2003): (1) *y; P{SUPor-P}Aldh^{KG02748} b c sp*: a stock with a *P* element construct (marked by *y*⁺) inserted in the *Aldh* 5' untranslated region, 169 bp from the start codon. The original insertion stock was generated by the BDGP gene disruption project (Bellen *et al.*, 2004); we introduced the markers *b*, *c* and *sp* and made the recombinant chromosome isogenic. In preliminary work, stock 1 had ALDH activity in the wild-type range, indicating that the insertion has little or no effect on *Aldh* expression. (2) *wg^{Sp}/CyO; ry⁵⁰⁶ Sb P{Δ 2-3}99B/TM6B,Tb*: a stock with the fixed *P* transposase source *P{Δ 2-3}*. (3) *y; wg^{Sp}/SM1,Cy; ry⁵⁰⁶*: a second chromosome balancer stock with the yellow body colour marker. (4) *Df(2L)N22-14/CyO*: a stock with a deficiency uncovering *Aldh* (breakpoints 29C1-2 and 30C8-9). (5) *CyO, Roi/In(2LR) bw^{VI}*: a second chromosome balancer stock wild-type for yellow.

Stock 1 females were crossed to stock 2 males, and the *y; CyO/P[y⁺] b c sp; Sb/+* male progeny were crossed to stock 3 females. From this cross, *y; b c sp/wg^{Sp}* male progeny that had lost the insert could be rapidly identified by the combination of yellow bodies and straight wings. These males were mated singly to stock 4 females, and the *Df(2L)N22-14/b c sp* progeny were used for DNA extraction and analysis to determine the nature of the excision event (see next section). If a line was to be retained, the *CyO/b c sp* male siblings of the analysed flies were crossed to stock 3 females, and the *SM1/b c sp* progeny crossed *inter se* to establish a *b c sp* stock.

(iii) Molecular and phenotypic characterization of lines

DNA was extracted from *Df(2L)N22-14/b c sp* flies using the Puregene DNA extraction kit (Gentra Systems). Lines which failed to produce a product when amplified with primers flanking the *P* element insertion site were retained for further analysis. The deletions in these lines were characterized by amplification with additional primer pairs, as described below. In addition, four lines in which the *P* element had excised precisely or nearly so were retained for use as controls.

ALDH enzyme activity of whole-fly homogenates was measured using a standard spectrophotometric assay, with 3 mM acetaldehyde as substrate. Detailed methods are given in Fry *et al.* (2004). Pyrazole (20 mM) was added to the reaction mixture to inhibit ADH, which would otherwise compete with ALDH for acetaldehyde. At least two replicate measurements were made per sex and line. Protein concentration of the homogenates was measured as described in Fry *et al.* (2004), and the results expressed as nM NAD⁺

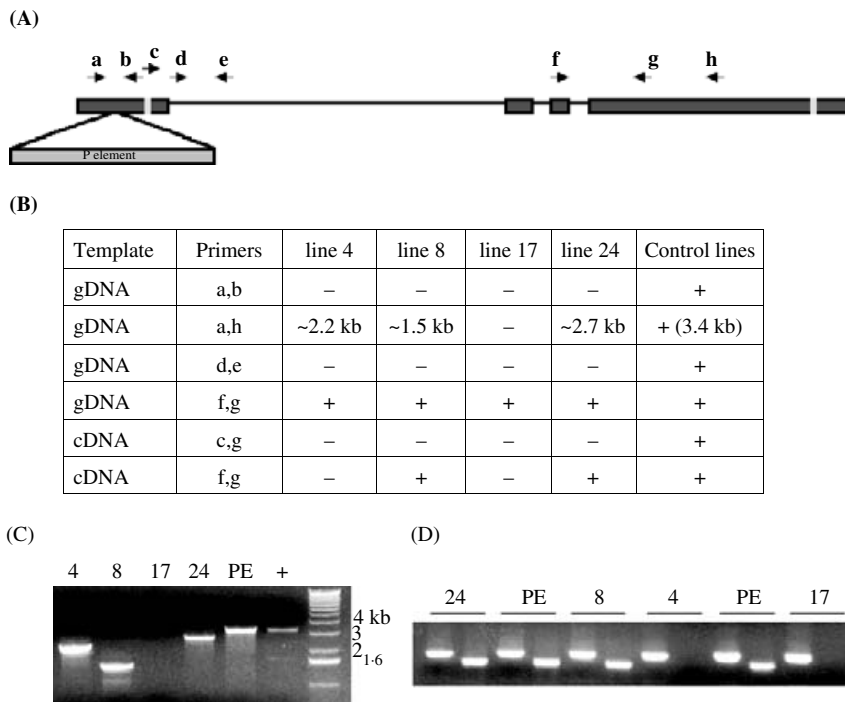


Fig. 1. (A) Diagram of *Aldh* transcribed region (4291 bp) showing approximate locations of primers used for the mutant screen. Start and stop codons are shown as gaps in the boxes. *P* element not to scale. (B) Amplification results with mutant and control lines (lines with precise excisions of the *P* element). +, product of normal size; –, no product; gDNA, genomic DNA; cDNA, complementary DNA. (C) Results of genomic DNA amplification with primers *a* and *h*. PE, precise excision (control) line; +, unrelated wild-type line. (D) Results of gDNA (first lane of pair) and cDNA (second lane) amplifications using primers *f* and *g*. Primer sequences (position of 5' base in genomic DNA, relative to first base of start codon): *a*, gttcttctgacagcactgt (–207); *b*, caaaactagaactgcgtctt (–63); *c*, gccaaagaactcgcagca (43); *d*, ggcattttagcaatcgcac (196); *e*, gaaatggggaagacctacac (381); *f*, ggtcgcctgctctaccgcctg (2313); *g*, ctcttcaccaactgggca (2799); *h*, tcaaaggattgcccacg (3173).

reduced per milligram total protein per minute, using the extinction coefficient of NADH at 340 nm of $6200 \text{ l mol}^{-1} \text{ cm}^{-1}$.

To check for *Aldh* expression, total RNA was extracted from adult males of the mutant and control lines using the RNeasy kit and QIASHredder columns (QIAGEN), with the addition of an extra DNase treatment. cDNA was synthesized using random hexamers (iScript cDNA synthesis kit, BioRad) and amplified using forward and reverse primers located in the second and third exons, respectively (see Section 3). In cases where no transcript was detected, amplification with primers from another gene confirmed that the cDNA synthesis had been successful.

For measurement of adult ethanol resistance, *b c sp* males from the mutant and control lines were crossed to stock 4 females. After two weeks, *Df(2L)N22-14/b c sp* (phenotypically wild-type) progeny were collected and placed in single-sex groups of 10 in shell vials containing normal medium. After 24 h, the flies were transferred without anaesthesia to vials containing cotton plugs moistened with 1 ml of a solution containing 3% sucrose and various concentrations of ethanol (0–12%). The vials were sealed with corks

Table 1. *Aldehyde dehydrogenase activity of mutant and control lines*

	Females	Males
Mutant lines	0.15 ± 0.08 (10)	0.74 ± 0.29 (9)
Control lines	2.73 ± 0.35 (11)	5.89 ± 0.71 (11)

Means \pm SEMs are shown, with sample sizes in parentheses. Units are nanomoles NAD^+ reduced per minute per milligram total protein.

and survivors counted after 24 h. At least one vial was set up per line, sex and ethanol concentration.

For measurement of larval ethanol resistance, *b c sp* males from two mutant and one control line were first crossed to stock 5 females. *CyO*, *Roi/b c sp* male progeny were backcrossed to stock 5 females, and *CyO*, *Roi/b c sp* virgin females and *In(2LR) bw^{VI}/b c sp* males collected, mated and allowed to lay eggs on apple juice–agar laying medium (see Fry, 2001 for detailed methods). Newly hatched larvae were placed in groups of 25 on medium containing either 0, 2%, 4% or 6% ethanol; the medium was in 35 mm Petri dishes, which were in turn taped to the bottoms of 100×20 mm Petri dishes. After 12 days, by which

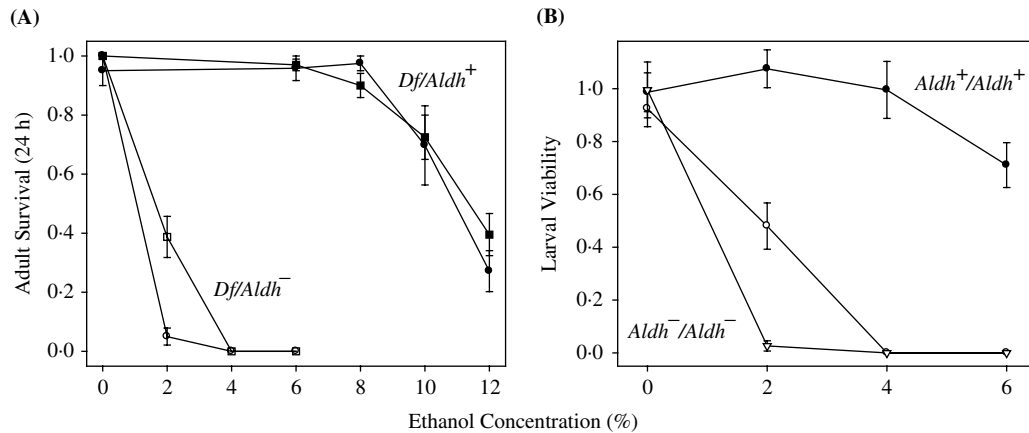


Fig. 2. (A) Adult ethanol resistance of *Aldh* null (–) and control (+) lines, tested over a deficiency which uncovers *Aldh*. Means and SEMs of pooled data from four mutant and four control lines are shown. Squares, males; circles, females. (B) Larval viability of two mutant (circles, line 17; triangles, line 24) and one control line on ethanol-supplemented media. Viability was measured as the observed number of *Aldh⁻/Aldh⁻* or *Aldh⁺/Aldh⁺* flies emerging divided by the number expected if all had survived.

time emergence had ceased, the dishes were frozen and flies counted. The viability of mutant and control flies was measured as the observed number of *b c sp* flies divided by 6.25 (=25/4), the expected number if all had survived. Eighteen dishes were set up per strain and ethanol concentration.

3. Results

A *P* element inserted in the 5' untranslated region of *Aldh* was mobilized to produce lines with precise and imprecise excisions. Four viable lines with unique deletions were isolated (Fig. 1). Failure of genomic DNA to amplify with primers *d* and *e* indicated that all four deletions extended into the first intron, thus removing the first 41 codons of the gene. Amplification with primers *f* and *g* indicated that the deletions did not remove the third and fourth exons. In three of the four lines, amplification with primers *a* (just upstream of the original insertion) and *h* (in the fourth exon) resulted in products of smaller-than-normal length by approximately 0.9–1.9 kb (Fig. 1C), consistent with the deletions terminating in the first intron. In the fourth line (line 17), no amplification was detected with primers *a* and *h*, suggesting that the deletion extended in both directions from the original insertion site. In addition to the four mutant lines, four lines producing normal-length fragments with primers *a* and *b* were recovered and maintained for use as controls.

Amplification of cDNAs with primers *f* and *g* showed that two of the mutant lines produced no *Aldh* transcript, while the other two produced a transcript lacking the coding region of the first exon (Fig. 1; confirmed by failure to amplify with primers *c* and *g*).

ALDH enzyme activity was measured in adults from each of the mutant and control lines. Analysis

of variance within each group (not shown) revealed no significant line effects or sex × line interactions ($P > 0.7$ in each case), so results from the different lines were pooled. Mutant flies had dramatically reduced ALDH activity, approximately 6% and 12% of control activity in females and males, respectively (Table 1). As we found previously for wild-type strains (Fry *et al.*, 2004), ALDH activity adjusted for total protein concentration is higher in males than females (Table 1).

The effects of the mutations on adult ethanol resistance was measured by placing the mutant and control chromosomes over a deficiency that uncovers *Aldh*. No significant variation among lines within each group was detected, so lines were again pooled for analysis. Mutant lines had dramatically reduced ethanol resistance compared with the controls (Fig. 2A), being completely killed by 4% ethanol, and surviving worse on 2% ethanol than the controls on 10% ethanol. On 2% ethanol, male mutants had significantly higher survival than females (Fig. 2A; $P < 0.05$, median test), possibly as a result of their higher ALDH activity (which presumably comes from the product of another gene). Otherwise, survival of the two sexes was similar.

Similarly, two mutant lines had much lower larval ethanol resistance than a control line (Fig. 2B). The mutant lines were completely killed by 4% ethanol, a concentration with no effect on survival of the control line. Although one mutant line had higher survival than the other on 2% ethanol, this is unlikely to be due to residual *Aldh* expression in the better-surviving line, because this line (line 17) was one of the two with no detectable *Aldh* transcript. There was no significant variation among the two mutant and one control lines in survival in the absence of ethanol (analysis of variance, $P > 0.8$).

4. Discussion

This report has three main findings. First, we have confirmed that *Aldh* is responsible for most (*c.* 90%) of the acetaldehyde dehydrogenase activity in adult *D. melanogaster*, as suggested by our earlier work with flies heterozygous for deficiencies covering the *Aldh* region (Fry *et al.*, 2004). This is important, because there are several other aldehyde dehydrogenases in the *D. melanogaster* genome (Sophos *et al.*, 2003). The products of these genes may be more effective catalysts with larger aldehyde substrates than with acetaldehyde, as is the case with several mammalian ALDHs (Vasiliou *et al.*, 2000), or may be produced in smaller amounts than the product of *Aldh*. Second, we have shown that *Aldh* null adults are poisoned by ethanol concentrations easily tolerated by wild-types. This is consistent with the results of Leal & Barbancho (1992), who showed that feeding an ALDH inhibitor to flies rendered them acutely sensitive to ethanol poisoning. Third, and most important, we have shown that *Aldh* null larvae are also extremely sensitive to ethanol, refuting the view that ALDH is relatively unimportant for ethanol metabolism in larvae (Heinstra *et al.*, 1989). In fact, for both larvae and adults, the ethanol sensitivity of *Aldh* nulls is comparable to that of *Adh* nulls (David *et al.*, 1976; Bijlsma & Bijlsma-Meeles, 1991).

Our results do not necessarily contradict the findings of Heinstra *et al.* (1989) and Heinstra & Geer (1991) that feeding an ALDH inhibitor to larvae had only a small effect on the flux of ethanol into lipid, for two reasons. First, these authors fed low concentrations of ethanol (around 1%) to third-instar larvae, while we exposed larvae to higher concentrations (2–6%) from hatching (the concentrations we used are nonetheless within the range encountered by wild *D. melanogaster*: McKenzie & McKechnie, 1979; Gibson *et al.*, 1981). Second, the effect of an enzyme on whole-body flux and on resistance are not necessarily the same. It is possible that ALDH lowers the concentration of acetaldehyde in a particularly sensitive tissue or organ, such as the nervous system (Deitrich, 2004), to below a critical threshold, without making a large contribution to flux at the level of the whole larva. Although we have not yet studied the tissue-specific pattern of *Aldh* expression, ALDH2 is expressed in the brain (as well as many other organs) in vertebrates (Sládek, 2003; Lassen *et al.*, 2005), and knockout mice lacking ALDH2 accumulate higher levels of acetaldehyde in the brain, for a given degree of alcohol consumption, than control mice (Isse *et al.*, 2002).

The *Aldh* null mutants are all viable and fertile, but we have found them to be more difficult to maintain as homozygotes than the control lines (data not shown). This is apparently due to reduced fertility,

because no difference was found between the mutants and controls in egg-to-adult viability in the absence of ethanol (Fig. 2B). The presence of fitness effects of *Aldh* null mutants even in the absence of ethanol is consistent with the high degree of conservation between *Drosophila* and mammalian ALDHs (e.g. 70% amino acid identity between *Drosophila Aldh* and human ALDH2). This conservation cannot be explained by the role of ALDHs in ethanol detoxification, because the split between arthropods and chordates preceded the emergence of fermentative yeasts by several hundred million years (Ashburner, 1998). Aldehyde dehydrogenases are believed to be important in detoxifying a large range of exogenous and endogenous aldehydes (Vasiliou *et al.*, 2000), which presumably accounts for their evolutionary conservation. The structural conservation and similar roles of ALDHs in ethanol detoxification in mammals and *Drosophila* stand in interesting contrast to the situation for alcohol dehydrogenases, which have evolved from different precursors in the two groups (Ashburner, 1998).

Our results suggest that *Aldh* may play as important a role as *Adh* in the evolution of ethanol resistance in *Drosophila*. Consistent with this possibility, we earlier showed (Fry *et al.*, 2004) that selecting *D. melanogaster* populations for ethanol resistance resulted in increases in ALDH activity (albeit significant only in females), as well as in ADH activity. We are currently investigating the role of *Aldh* in the large difference in ethanol resistance between temperate and tropical populations of *D. melanogaster* (David & Bocquet, 1975; David *et al.*, 1986; Parkash *et al.*, 1999).

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