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# Isolation of *Caenorhabditis elegans* Mutants Lacking Alcohol Dehydrogenase Activity

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*Alcohol dehydrogenase (ADH) and the genes encoding this enzyme have been studied intensively in a broad range of organisms. Little, however, has been reported on ADH in the free-living nematode* Caenorhabditis elegans. *Extracts of wild-type* C. elegans *contain ADH activity and display a single band of activity on a native polyacrylamide gel. Reaction rate for alcohol oxidation is more rapid with higher molecular weight alcohols as substrate than with ethanol. Primary alcohols are preferred to secondary alcohols.* C. elegans *is sensitive to allyl alcohol, a compound that has been used to select for ADH-null mutants of several organisms. Allyl alcohol-resistant mutant strains were selected from ethylmethanesulfonate (EMS)-mutagenized nematode populations. ADH activity was measured in extracts from eight of these strains and was found to be low or nondetectable. These results form a basis for molecular and genetic characterization of ADH expression in* C. elegans.

**KEY WORDS:** alcohol dehydrogenase; allyl alcohol selection; ADH$^-$ mutants; *Caenorhabditis elegans.*

## INTRODUCTION

Alcohol dehydrogenase (ADH; EC 1.1.1.1) catalyzes the reversible oxidation of ethanol to acetaldehyde coupled with reduction of $NAD^+$. The proteins and genes encoding ADH have been intensively studied in a broad range of organisms including yeast, maize, horse, and fruit flies (Ciriacy, 1975; Williamson *et al.,* 1980; Williamson and Paquin, 1987; Freeling and Birchler, 1981; Jornvall *et al.,* 1987; O'Donnell *et al.,* 1975; Goldberg *et al.,*

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1983; Chambers, 1988). Surprisingly, very little has been reported about this enzyme in the free living nematode *Caenorhabditis elegans* even though the genetics and molecular development of this organism have been extensively characterized (Brenner, 1974; Herman, 1988; Sulston *et al.,* 1983). ADH activity had been reported to occur in a nonidentified *Caenorhabditis* species (Cooper and Van Gundy, 1971) and in *C. elegans* (Bolla *et al.,* 1987). We were not able to find reports of further characterization of ADH in this nematode.

ADH functions in both the production and the degradation of alcohols. In yeast and maize, ADH catalyzes the final step in the production of ethanol (and $NAD^+$) as the end product of glycolysis during anaerobic metabolism. Maize and yeast mutants lacking ADH activity are unable to survive anaerobic conditions because they are can not regenerate the $NAD^+$ required for anaerobic glycolysis (Ciriacy, 1975; Freeling and Birchler, 1981). The levels and regulation of this enzyme and its role in ethanol tolerance have been extensively studied in *Drosophila,* where ADH functions to catalyze the first step in the utilization or detoxification of ethanol and other alcohols (reviewed by Chambers, 1988). ADH may function in both anaerobic survival and ethanol utilization in nematodes. Cooper and Van Gundy (1971) found that, in microaerobic and anaerobic environments, *Caenorhabditis* spp. produce ethanol, suggesting that ADH has a role in anaerobic metabolism. The "beer mat" nematode, *Panagrellus redivivus,* reported to tolerate up to 10% ethanol, also displays ADH activity (Kriger *et al.,* 1977). In this species, ADH and aldehyde dehydrogenase activity are coinduced by addition of ethanol and ADH appeared to be required for the ethanol tolerance.

A major factor contributing to the utility of ADH for studies of gene expression and regulation is the availability of simple selections for and against expression of this enzyme. In yeast and in maize and other plants, allyl alcohol has been used to select mutants that do not express ADH activity (Ciriacy, 1975; Wills and Phelps, 1975; Freeling and Birchler, 1981; Jacobs *et al.,* 1988). Allyl alcohol is converted by ADH to the toxic compound acrolein; thus only organisms not expressing ADH survive. A similar strategy, using the secondary alcohols penten-3-ol or pentyn-3-ol, has been used to obtain ADH-null mutants in *Drosophila* (Sofer and Hatkoff, 1972; O'Donnell *et al.,* 1975). In yeast and maize, selection for revertants expressing ADH activity is based on the observation that ADH-null mutants cannot grow anaerobically. *Drosophila* revertants, on the other hand, have been selected by their ability to survive exposure to ethanol (Goldberg *et al.,* 1983).

Interest in understanding the metabolism of free-living and plant-parasitic nematodes is increasing as sustainable agriculture requires in-

creased knowledge of the soil ecosystem. Despite the major accomplishments in understanding the development and genetics of *C. elegans,* knowledge about the genetics of regulation of metabolism in this organism is limited (Bolla, 1980; Wadsworth and Riddle, 1989; O'Riodan and Burnell, 1989). The present work was initiated to provide a basis for genetic and biochemical investigation of the regulation and role in metabolism of ADH in the genetically tractable nematode *C. elegans.*

## MATERIALS AND METHODS

### Caenorhabditis elegans Strains and Maintenance

The wild-type strain of *C. elegans,* var. Bristol strain N2, used in these experiments was obtained from the *Caenorhabditis* Genetic Stock Center (Division of Biological Sciences, University of Missouri). Mutant and wild-type strains were maintained on bacterial lawns on NG agar plates as described by Brenner (1974).

For allyl alcohol selection, plates were prepared by cooling NG agar to approximately 50°C, then adding allyl alcohol (Gold Label, Aldrich Chemical Co.), with stirring in a fume hood, to the indicated concentration. Plates were poured in a fume hood and stored in sealed plastic bags. Because allyl alcohol is volatile, plates were sealed with Parafilm during selection.

### Mutagenesis

*C. elegans* mutations were generated by treatment with ethylmethanesulfonate (EMS) as described by Brenner (1974). Nematodes enriched for young adults were mutagenized for 4 hr in 0.1 *M* EMS. About 25 nematodes were transferred to each of 30 NGM plates and allowed to reproduce by self-fertilization for two generations to obtain worms homozygous for recessive mutations. Nematodes were then transferred to NGM plates with 0.3% (v/v) allyl alcohol. Survivors were picked after 2–3 days and then transferred to NG plates. After growth on NG plates, nematodes were retested on allyl alcohol plates. All mutations are derived from clonally propagated populations and are independent isolates.

### Preparation of Extracts

*C. elegans* was grown on *Escherichia coli* on NG plates until the bacterial lawn had just cleared. Nematodes were collected and separated from remaining *E. coli* essentially as described by Sulston and Hodgkin (1988). Nematodes were washed from a 9-cm NG plate into 5 ml of M9 medium

(Brenner, 1984), mixed with an equal volume of 60% (w/w) sucrose, then centrifuged for 3 min at 2000 rpm. Nematodes floating on the sucrose solution were collected, diluted to 4 ml with 0.1 *M*Tris–HCl, pH 8.0, pelleted by centrifugation (4000 rpm, 5 min), then resuspended in 4 ml of 0.1 *M*Tris–HCl, pH 8.0, and centrifuged. Pellets were frozen until needed at −70°C. For extraction, pellets were thawed, then disrupted by vortexing at high speed for 2 min with 0.2 g glass beads (0.45–0.50 mm in diameter). Extract was transferred to 1.5-ml microcentrifuge tubes and centrifuged (14,000 rpm, 3 min) to remove cell debris.

### ADH Activity

ADH activity was determined spectrophotometrically using assay conditions essentially as described for yeast ADH (Lutstorf and Megnet, 1968). Assay conditions were 32 m*M* sodium pyrophosphate buffer, pH 8.8, 5 m*M* $NAD^+$, and 0.2 *M* ethanol or other alcohol, as indicated. Change in absorbance at 340 nm was measured. Activity is expressed as milliunits (mU; millimoles $NAD^+$ converted to NADH per minute). Protein concentration was determined using the Biorad microassay as recommended by the manufacturer using bovine standard albumin (BSA) as the protein standard.

### Isozyme Electrophoresis

Acrylamide gel electrophoresis was carried out using the Pharmacia-LKB PhastSystem. A 10 to 15% gradient gel was used and electrophoresis was at 400 V for 30 min. The gel was then stained for ADH activity (Fowler *et al.*, 1972). After staining for 5 min at 25°C, gels were rinsed in water, soaked in glycerol–acetic acid–water (1:1:8), then air-dried.

## RESULTS

### Alcohol Dehydrogenase Activity

The ADH level in extracts of *C. elegans* strain N2 averaged 186 mU/mg total protein (Table I). Higher activity values correlated with the presence of a greater proportion of adult hermaphrodites in the extract, possibly reflecting developmental regulation of ADH activity. ADH enzymes have different relative reaction rates using various alcohols (Williamson and Paquin, 1987). The ADH of *Drosophila* spp. differs from that of other organisms in its preference for secondary alcohols (Winberg *et al.*, 1982; reviewed by Chambers, 1988). The ADH activity levels of *C. elegans* extracts was determined using as substrate primary alcohols (ethanol, 1-propanol, and 1-butanol) and

**Table I.** Alcohol Dehydrogenase Activity in *C. elegans* Strains[a]

| Strain | ADH activity (mU/mg protein) | |
|---|---|---|
| N2 | 186 ± 85 | |
| | Extract 1 | Extract 2 |
| AL1 | 0 | 99 |
| AL2B | 0 | 11 |
| AL6 | 0 | 0 |
| AL9B | 15 | 0 |
| AL10 | 0 | 0 |
| AL11 | 0 | 0 |
| AL12 | 0 | 0 |
| AL13 | 0 | 0 |
| AL14 | 0 | 0 |

[a]Four independent extractions were carried out for the wild-type strain N2. The average activity followed by the standard deviation is presented. Two independent extractions were carried out in separate experiments for each mutant. The ADH activity determined for each experiment is presented. The detection limit for ADH activity was about 5 mU/mg protein.

secondary alcohols (2-propanol and 2-butanol). Results are shown in Table II. Reaction rates increased with the molecular weight of the alcohol and were higher for primary than for secondary alcohols of the same molecular weight.

Extracts from *C. elegans* N2 were electrophoresed on nondenaturing polyacrylamide gels and stained for alcohol dehydrogenase activity. Mobility was compared to that of yeast ADH (Fig. 1). A single band of activity that migrates slightly further than the most rapidly migrating yeast ADH band

**Table II.** Substrate Specificity of *C. elegans* ADH

| Substrate | Relative reaction rate[a] |
|---|---|
| Ethanol | 100 |
| 1-Propanol | 254 ± 98 |
| 2-Propanol | 193 ± 53 |
| 1-Butanol | 468 ± 180 |
| 2-Butanol | 210 ± 79 |

[a]Reaction rates are presented as a percentage of the rate obtained using ethanol as substrate. Activity was determined using crude extracts of *C. elegans* strain N2. Three separate experiments were carried out. The average relative reaction rate with standard deviation is presented for each substrate. Substrates were present at a final concentration of 0.2 *M*.

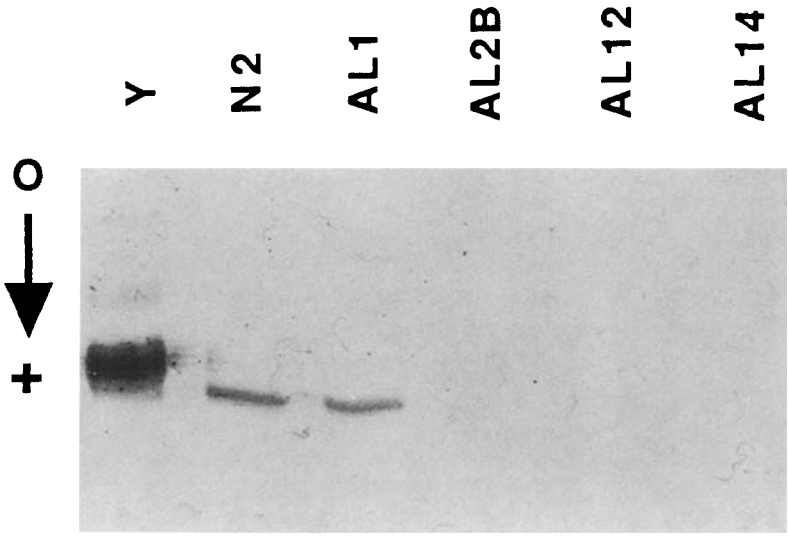


**Fig. 1.** Native polyacrylamide gel showing ADH activity. Yeast ADH (Sigma Chemical) is shown in lane 1 (Y); extract from wild-type *C. elegans* (N2) is shown in the second lane; and extracts from the allyl alcohol mutant strains AL1, AL2B, AL12, and AL14 are shown in the remaining lanes. The total protein concentration of each crude extract was measured and extracts were diluted to equal concentration (2 mg total protein/ml) before application to the gel.

was seen when ethanol was used as substrate. The same mobility relative to yeast ADH was observed after electrophoresis and staining of crude extracts on cellulose acetate membrane (not shown). Since enzymes migrate primarily by charge in native polyacrylamide and cellulose acetate gels, the *C. elegans* enzyme is apparently quite acidic. The presence of a single activity band suggested that one isozyme of ADH, and thus most likely one major gene, is present in *C. elegans.*

### *C. elegans* Mutant Isolation and Characterization

The selective agent allyl alcohol (propene-3-ol) has been used to isolate ADH-null mutants from a number of different species. The secondary alcohols pentene-3-ol and pentyne-3-ol have been used to select for ADH-null mutants in *Drosophila* (Sofer and Hatkoff, 1972; O'Donnell *et al.,* 1975). Because *C. elegans* preferentially utilizes primary alcohols as substrates, allyl alcohol was chosen as the selective agent for these experiments. NG plates containing a range of concentrations of allyl alcohol were prepared and inoculated with *E. coli* OP50. Allyl alcohol, at the concentrations used, did not appear to affect the growth of the *E. coli.* Approximately 1000 nema-

todes were applied to each plate and the survival of nematodes at each concentration was determined after 2 days (Fig. 2). The majority of nematodes is killed at 0.01% allyl alcohol. At this concentration larval stages survive and some recovery is observed with time, perhaps due to the volatility of the allyl alcohol. At 0.3% allyl alcohol adult nematodes cease to move within 30 min. Eggs are not killed but hatch into larvae. Newly hatched L1 larvae are not immobilized as are older stages, but become immobilized as they develop into L2 larvae. Nematodes surviving on 0.3% allyl alcohol plates after 2 days were transferred to NG plates and allowed to reproduce clonally by self-fertilization. The resulting populations were retested for allyl alcohol resistance. Only one spontaneous mutant, AL1, survived replating on 0.3% allyl alcohol.

EMS mutagenesis was carried out as described under Materials and Methods to obtain additional allyl alcohol-resistant nematode lines. Mutation frequency was about that expected for a single-copy gene [$5 \times 10^{-4}$ (Brenner, 1974)]. Twelve independently derived strains were identified that survived repeated plating on 0.3% allyl alcohol. Eight of these strains were investigated in more detail. To eliminate the possibility that allyl alcohol resistance was due to contamination or mutation of the microbial food source, eggs of each mutant strain were sterilized by treatment with alkaline hypochlorite (Sulston and Hodgkin, 1988), then plated onto a fresh lawn of *E. coli* OP50. Allyl alcohol resistance was not altered by this treatment. Extracts of each strain, grown on nonselective (NG) plates, were assayed spectrophotometrically for ADH activity. ADH activity was greatly reduced

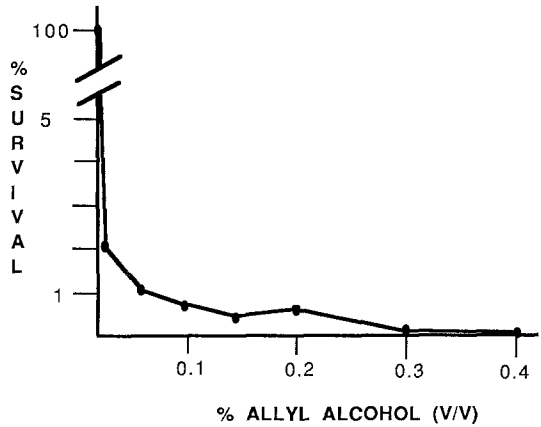


**Fig. 2.** Effect of different allyl alcohol concentrations on the survival of *Caenorhabditis elegans* N2. Survivors were scored as the percentage of nematodes able to move after 2 days on NG plates containing the indicated concentrations of allyl alcohol.

or not detected in the EMS-derived mutants (Table I). Native acrylamide gel electrophoresis was carried out on extracts of these mutants and little, if any, ADH activity was detected compared to the wild type (Fig. 1). Extracts of the spontaneous allyl alcohol-resistant mutant strain, AL1, contained no ADH activity in one experiment and wild-type ADH activity in a second experiment (Table I). A band corresponding to ADH and with the same mobility as the wild-type was seen in this extract of AL1 (shown in Fig. 1). Plating of subcultures of AL1 onto allyl alcohol revealed that the stock cultures had reverted to allyl alcohol sensitivity, raising the possibility that the mutation in AL1 was unstable.

Upon prolonged staining a second activity band with lower mobility was occasionally observed in some extracts of wild-type and mutant nematodes (see lane 6 in Fig. 1), but this band did not correlate with the presence of spectrophotometrically determined ADH activity levels. A possible explanation is that this sporadic activity band is due to ADH activity of bacteria remaining in the nematode gut and that this activity was too low to be detected spectrophotometrically.

Microscopic examination of the allyl alcohol-resistant mutants revealed no obvious difference from the wild type in appearance or behavior except for strain AL14. Growth of cultures of this strain was notably slow. The generation time of this mutant was determined by microscopic observation of the time required for eggs to develop into egg-laying hermaphrodites. Egg laying in strain AL14 was not observed until 5 days after hatching, compared to 3 days for the wild-type strain N2.

## DISCUSSION

*C. elegans* has been shown in this work and in the work of others to contain alcohol dehydrogenase activity. This paper establishes the groundwork for using ADH to investigate metabolism and gene regulation in *C. elegans.* We have demonstrated that allyl alcohol is a very specific selective agent for isolating null mutations in ADH expression. The eight EMS-induced allyl alcohol-resistant mutant strains tested had reduced ADH activity. The frequency at which allyl alcohol-resistant ADH null mutations were obtained after EMS mutagenesis and the concurrent loss of the single band of ADH activity on an isozyme gel are consistent with the existence of a single major gene for ADH in *C. elegans.* The ease and specificity of this selection scheme indicate that allyl alcohol selection should be a useful agent to investigate mutation frequency. For example, the ability to select for or against ADH expression has been used as a tool to study transposition events in maize and yeast (Bennetzen *et al.,* 1984; Paquin and Williamson,

1984, 1986) and may also prove useful for studying transposition in *C. elegans.* The lack of an obvious phenotype, besides allyl alcohol resistance, for most of the mutants lacking ADH activity indicates that this gene is not required for growth under standard laboratory conditions, perhaps making allyl alcohol resistance a useful strain marker.

The results presented here raise interesting, and now approachable, questions about ADH in *C. elegans.* The number of different genetic loci conferring allyl alcohol resistance is yet to be determined, although from work in other systems, it is likely that at least some of the mutants are in the ADH structural gene. Of particular interest is the allyl alcohol-resistant mutant AL14 that lacks ADH activity and has a longer life cycle than does the parent strain. The slow growth of AL14 could be due to a second mutation linked to the lesion conferring the ADH null phenotype or to a lesion in a regulatory gene that affects the expression of other genes as well as ADH. If the second possibility is true, this mutation should provide insights into metabolic regulation in nematodes. The presence of an apparent revertant of AL1 which has regained ADH activity and lost allyl alcohol resistance is also intriguing. The spontaneous appearance of this putative revertant raises the possibility that the mutation in AL1 is unstable in the absence of selection. Instability is a characteristic of transposon insertion mutations, one of the most common types of spontaneous mutation (Paquin and Williamson, 1984; Moerman and Waterston, 1989).

The data presented here demonstrate the potential utility of the ADH system for investigation of regulation of metabolism in nematodes. Two observations suggest that ADH is developmentally regulated in *C. elegans.* The first observation is that ADH levels are higher in populations with a high proportion of adults. The second is the relative tolerance to allyl alcohol of eggs and L1 larval stages. These results predict that ADH activity increases during larval development. A major shift in energy metabolism occurs during the transition from the L1 to the L2 stage of development (Wadsworth and Riddle, 1989). At this stage there is a major decrease in glyoxylate cycle enzymes and an increase in TCA enzymes. The ability to select for mutations with altered ADH expression may provide an inroad to exploring the coordinate regulation of these enzymes. The most direct approach to answering many of the questions raised by this work would be to obtain a clone of ADH. Transposon mutagenesis has proved useful for cloning several genes of interest in *C. elegans* (Moerman *et al.,* 1986; Collins *et al.,* 1987). The results of the allyl alcohol selection presented here indicate that transposon mutagenesis may be a viable approach to obtaining a clone of *C. elegans* ADH.

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