Functional Alcohol Dehydrogenase Mutants of *Saccharomyces cerevisiae*
Conferring Temperature-Conditional Allyl Alcohol Resistance

John G. Hall and Christopher Wills

Department of Biology, C-016, University of California at San Diego, La Jolla, California 92093

Manuscript received February 10, 1986
Revised copy accepted September 11, 1986

**ABSTRACT**

Selection for allyl alcohol resistance in respiratory incompetent yeast is a highly specific method for isolating functional mutations at *ADH1*, the gene coding for the cytoplasmic alcohol dehydrogenase, ADH1. Because of the nature of this selection scheme, the ADH1 activity of such mutants is retained, but the kinetic characteristics of the enzymes are altered. The high specificity for targeting functional mutations at this locus suggested that selection for enzyme variants with more subtle phenotypic effects might be possible. Here, we describe functional ADH1 mutants that are temperature-conditional in their allyl alcohol resistance. Haploid cells of one of these mutants grow well on plates at 10 mM allyl alcohol at 19°, but not at 37°, the restrictive temperature. A second mutant grows well at 10 mM at 37°, but its growth is restricted at 19°. What distinguishes these mutants from other temperature-sensitive mutants is that the temperature-conditional growth phenotypes described here must be due to interactions between allyl alcohol levels and ADH1 functional properties and cannot be due to lability of the enzyme at the restrictive temperature. This system shows promise for the investigation of functional enzyme variants that differ by only one or two amino acid residues but have significant temperature- and substrate-conditional effects on growth phenotypes in both the haploids and the diploids.

Several laboratories have developed model systems for investigating how the metabolic machinery of microorganisms may be altered by selection pressure for utilization of novel carbon sources (for reviews, see Mortlock 1984). The responses in such evolution "experiments" are remarkably diverse, and consequently, these systems have become useful for modeling the evolution of metabolic pathways and the adaptation of new enzyme functions from preexisting ones. Changes in an enzyme's substrate specificity are a common selective outcome, as in the "evolved" β-galactosidase system of *E. coli* (Hall 1984) and the amidase system of *Pseudomonas aeruginosa* (Clarke 1984). Another experimental system for studying evolution at the biochemical level is the alcohol dehydrogenase system of yeast. It differs from other selection schemes in being remarkably specific for the selection of functional mutations at a single enzyme locus, *ADH1*.

In *Saccharomyces cerevisiae*, two genes, *ADH1* and *ADH2*, code for the two cytoplastically expressed alcohol dehydrogenases, ADH1 and ADHII (Giriacy 1975; gene nomenclature revised by Taguchi, Giriacy and Young 1984). ADH1 is largely a constitutively expressed enzyme, whereas ADHII is repressed by glucose and in anaerobically grown or petite cells. This and the kinetics of the two cytoplasmic isozymes suggest that ADHII is involved in ethanol oxidation and ADH1 is involved primarily in ethanol production during fermentation (Wills 1976a). Yeast strains that cannot respire aerobically generate energy for growth by glycolysis alone. Because yeast strains having no cytoplasmic ADH activity cannot survive as petites, the presence of a functional cytoplasmic ADH (i.e., ADH1) is apparently essential for survival under these conditions, since it is largely responsible for regenerating NAD+(H+) in glycolysis (Wills and Phelps 1975; Wills, Kratofil and Martin 1982).

Taking advantage of this fact, Wills developed a scheme for selecting large numbers of functional mutants at *ADH1* (Wills and Phelps 1975; Wills 1976b). The basis for selection is the fact that allyl alcohol (2-propen-1-ol), when added to growth media, is oxidized by ADH1 to acrolein (acrylaldehyde, 2-propenal), a toxic aldehyde that inactivates various proteins, including yeast ADH (Rando 1974), binds to nucleic acids (Izard and Liberman 1978) and kills the cell. The only cells that survive are those that are able to minimize the toxic effects of acrolein. A large proportion (up to 40% in some experiments) of the allyl-alcohol-resistant mutants obtained in these experiments involve mutations in *ADH1* in which the activity of the enzyme is retained (Wills 1976b; Wills and Jornvall 1979). This suggests that there are a large number of ways in which yeast can respond to this environmental challenge via alterations in this particular gene.

The mechanism of resistance involves changes in
the kinetic properties of the enzyme that, in turn, lead to increases in the ratio of NADH to NAD⁺ in the cytoplasm (WILLS 1976a; WILLS and PHELPS 1978). Since acrolein is apparently not metabolized further in the cell (WILLS and PHELPS 1978), this drives the reaction toward the harmless alcohol, decreasing acrolein levels.

The specificity of this selection scheme for targeting functional mutations to this gene makes it extremely useful for investigating the structural and functional nature of single amino acid substitutions that leave the major function of the molecule intact but produce small changes in its kinetic parameters. Questions to be addressed include whether structural and functional constraints exist on the range of "adaptive" amino acid substitutions that confer allyl alcohol resistance. For example, do resistant substitutions occur in the neighborhood of the active site, or are they distributed throughout the molecule? Is resistance achieved by means of a limited number of kinetic changes, or can the same functional result be achieved in a variety of ways? Preliminary answers are now available for some of these questions [See WILLS (1984) for review]. The five functional substitutions that have been localized are scattered throughout the ADHI subunit.

The specificity of allyl alcohol selection also suggests potential for selecting ADHI mutants that are even more subtle in their phenotypic effects, i.e., in which allyl alcohol resistance is conditional upon some aspect of the physical or chemical cellular environment. Much comparative biochemical work does suggest, in fact, that the functions of enzymes can be changed via natural selection by the thermal, hydrostatic pressure, and intracellular solute environments of the organisms in which they are found (HOCHACHKA and SOMERO 1984). Here, we describe ADHI functional mutants that confer temperature-conditional allyl alcohol resistance.

MATERIALS AND METHODS

A spontaneous cytoplasmic petite was isolated from wild-type strain S288C (a mating type) and subcloned. Homogenates of cells from this clone (HP-73-4) grown in batch culture at 30°C, showed only ADHI activity on starch gels following electrophoresis and did not grow on complete medium (2% Bacto-peptone, 1% yeast extract, w/v from Difco) with 2% glycerol (v/v) sterile ethanol and 5 mM allyl alcohol or 0.4% freshly distilled sterile acetaldehyde + 1 ppm Antimycin A to convert the cells to phenotypic petites. Duplicate plates were incubated at 19°C and 37°C along with control plates from which allyl alcohol had been omitted.

To measure the growth of haploid cells, three individual cells from each of four allyl-alcohol-resistant segregants were pulled by micromanipulation onto fresh agar slabs of the following media: (1) YEPD + 0.1, 2.5, 5 or 10 mM allyl alcohol + 1 ppm Antimycin A; (2) YEPD + 0.5, 10, 15, or 20 mM allyl alcohol + 1 ppm Antimycin A; (3) YEPD + 0.1, 2.5, 5 or 10 mM allyl alcohol + 0.4% freshly distilled sterile acetaldehyde + 1 ppm Antimycin A. Cells for each experiment were first grown in 5 ml of the appropriate liquid medium without allyl alcohol for 16 hr at 30°C. The agar slabs were transferred to plates of identically prepared media, which were then sealed with paraffin film and incubated at 19°C or 37°C. Growth of resistant segregants was monitored by measuring clone diameter at least daily with a calibrated ocular micrometer. Growth experiments involving diploids were conducted in the same manner.

RESULTS

Selection and genetic analysis of ADHI mutants: The proportion of allyl-alcohol-resistant mutants obtained in selection experiments at 19°C and 37°C with electrophoretically altered ADHIs is comparable to earlier results at 30°C (WILLS, KRATOFIL and MARTIN 1982). Of 47 resistant mutants, 21—approximately 45%—have ADHs that are electrophoretically distinguishable from the wild type. Nine of these are more positively charged and 12 are more negatively charged than wild-type ADHI. In backcrosses involving mutants segregating 2:2 for allyl alcohol resistance, the altered electrophoretic phenotype always cosegregated with resistance, indicating probable allelism of allyl alcohol resistance with ADHI.

Some of the other resistance mutants with wild-type ADHI electrophoretic phenotypes also appear to be
TABLE 1
Genetics of allyl-alcohol-resistant mutants of Saccharomyces cerevisiae

<table>
<thead>
<tr>
<th>Mutant</th>
<th>No. of tetrads</th>
<th>Growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADHI mobility relative to wild type</td>
<td>AOH resistance segregation</td>
</tr>
<tr>
<td></td>
<td>2:2 segregation</td>
<td>19°</td>
</tr>
<tr>
<td>EAA5-19-F3</td>
<td>1.04</td>
<td>6</td>
</tr>
<tr>
<td>EAA5-37-S7</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>EAA5-19H1-S6</td>
<td>0.96</td>
<td>25</td>
</tr>
<tr>
<td>EAA5-19-S10</td>
<td>1.00</td>
<td>—</td>
</tr>
<tr>
<td>EAA5-37H1-S7</td>
<td>0.80</td>
<td>52</td>
</tr>
<tr>
<td>EAA5-37-S4</td>
<td>0.96</td>
<td>17</td>
</tr>
</tbody>
</table>

* Number of screened tetrads segregating 2:2 for electrophoretic mobility changes and for allyl alcohol resistance. Allyl alcohol resistance cosegregated 2:2 with ADHI electrophoretic mobility segregation in all screened tetrads.

Mutant EAA5-37-S7 (hereafter referred to as HTR1) was obtained independently under similar conditions, but its ADHI is electrophoretically indistinguishable from wild type. Thirty-three of 34 tetrads segregated 2:2 for allyl alcohol resistance. All segregants (40 tetrads) of a cross of a resistant HTR segregant to a resistant mobility mutant (Rf = 0.96) were resistant at 5 or 10 mM. This indicates that the resistance gene is within ±1.2 cM of ADHI. In contrast to LTR1, HTR1 is resistant at 37°, but markedly less so at 19°, to 10 mM allyl alcohol.

**Growth characteristics of LTR1 and HTR1 strains:** The two haploid mutants grow well in the absence of allyl alcohol and exhibit comparable growth characteristics at both temperatures (Figure 1). On YEPDE + Antimycin A media at 37° the growth constant, k, for increase in clone diameter (~22–70 hr) is 0.087 ± 0.0016 hr⁻¹ (±SEM; n = 3) for wild type, 0.082 ± 0.0009 hr⁻¹ (n = 8) for HTR, and 0.043 ± 0.0020 hr⁻¹ (n = 7) for LTR. At 19°, k equals 0.047 ± 0.0030 hr⁻¹ (n = 3), 0.048 ± 0.0004 hr⁻¹ (n = 8), and 0.037 ± 0.0008 hr⁻¹ (n = 5), respectively. Ethanol (2%) added to the medium consistently depresses growth of mutants and wild type, but substitution of 0.04% acetaldehyde abolishes any growth-rate differences among wild type and mutants at either temperature. Added acetaldehyde has no effect on the growth characteristics of the wild-type strain.

The temperature-conditional growth characteristics only become apparent when allyl alcohol is added to the media. The temperature sensitivity of resistance is most evident in the presence of added ethanol at 10 mM allyl alcohol (Figure 2). At 19°, HTR1 plateaus at a maximum colony size of only about 240 μm, whereas LTR1 grows to ≥2000 μm under these conditions. Increasing the allyl alcohol concentration results in progressive depression of growth (Figure 3). At 19°, LTR1 is not appreciably affected by allyl alcohol until above 10 mM, whereas significant depression of HTR1 growth is noted above 5 mM. At 37°, increasing allyl alcohol concentrations progressively decrease the exponential growth rate of HTR1. LTR1, however, is extremely sensitive to allyl alcohol at this temperature and shows no cell division at 10 mM or higher (Figure 3C).

Although depressing the growth of the ADHI mutant strains, ethanol provides protection against the deleterious effects of allyl alcohol. Conversely, acetaldehyde stimulates growth but increases susceptibility

**mutations at this locus.** Three resistant mutants with electrophoretically wild-type ADHIs were backcrossed to S288C and were found to segregate 2:2 for allyl alcohol resistance at 5 mM. Crosses involving a resistant segregant from each of these crosses, and a resistant ADHI electrophoretic mutant segregant, yielded tetrads in which segregants were all resistant (13–40 tetrads). This result is expected if the genes conferring allyl alcohol resistance are allelic, or very tightly linked, to ADHI. This and earlier work thus suggest that many of the nonelectrophoretic mutants are also ADHI functional mutants and that the efficiency of the selection scheme for ADHI mutants must be very high, probably substantially greater than 50%.

Some of the ADHI electrophoretic and nonelectrophoretic mutants consist of two classes, based upon the temperature restrictiveness of allyl alcohol resistance (Table 1). The first class, backcrossed 2–3 times to S288C, grows well at 37° but poorly at 19° on 10 mM allyl alcohol plates, compared to controls lacking allyl alcohol. These are designated HTR (high-temperature-resistant) mutants. The second class grows well at 19° but poorly at 37° under the same conditions (LTR, low-temperature-resistant mutants). Other allyl-alcohol-resistant mutants show little or no temperature sensitivity.

Two allyl-alcohol-resistant mutants with contrasting temperature sensitivities were characterized in more detail. Mutant EAA5-37H1-S7 (hereafter referred to as LTR1) was initially isolated at 5 mM allyl alcohol at 37°, following saccharin mutagenesis. Its ADHI has an electrophoretic mobility of 0.80 relative to wild type. The allyl alcohol resistance of LTR at 5–15 mM cosegregated 2:2 with the altered electrophoretic phenotype in 32 tetrads, indicating that the resistance is within ±1.5 cM of ADHI and probably coincident with the ADHI structural gene mutation (MORTIMER and HAWTHORNE 1969). Interestingly, this ADHI mutant, although selected at 37° and 5 mM allyl alcohol, is resistant to 10 mM allyl alcohol at 19°, but not at 37°.
to allyl alcohol. These effects are manifested in growth rate and in the maximum diameter the colony can attain before growth ceases. The effect on maximum diameter is shown in Table 2. Temperature-dependent allyl alcohol resistance decreases markedly in the absence of ethanol, the mutants being able to divide only a few times at 10 mM before growth ceases entirely. Added acetaldehyde, if anything, depresses resistance even further; limited temperature-conditional allyl alcohol resistance is apparent only at 2.5 mM. With no additions to the media, HTR1 growth ceases at ~100 μm at 19°C, but LTR1 continues to grow throughout the 7- to 9-day period of measurement (Figure 4; Table 2)—a difference that is more pronounced with acetaldehyde. At 37°C there is little difference between the two strains. HTR1 shows significantly greater growth on YEPD alone, but both strains grow identically with added acetaldehyde, with growth stopping below 300 μm (Figure 4; Table 2).

Neither the S288C (wild-type) diploid nor either of the mutant/wild-type heterozygotes grows at all under these conditions. These observations provide additional evidence for presuming that HTR1 is, in fact, allelic to LTR1. These relationships are also apparent at other allyl alcohol concentrations.

**DISCUSSION**

The isolation in this study of temperature-sensitive, allyl alcohol resistance mutants demonstrates that in yeast it is possible to select for subsets of ADHI mutants with conditional phenotypic effects. Since they grow well at both high and low temperatures in the absence of allyl alcohol, the temperature-conditional resistance phenotypes of the two mutants characterized here must be attributed to interaction between allyl alcohol levels and ADHI functional properties and cannot be due merely to differences in thermolability. This fact distinguishes these mutants from other cases of temperature sensitivity, which are typically characterized by enzyme dysfunction or denaturation at the restrictive temperature (e.g., allyl-alcohol-resistant mutants of *E. coli* with thermolabile alcohol dehydrogenase; Lorowitz and Clark 1982).

One of the more interesting aspects of this study is the degree to which changing the chemical environment (i.e., addition of ADH substrates) affects the growth of mutant strains as well as their resistance—or lack of it—to allyl alcohol at different tempera-
Yeast ADHI Functional Mutants

3.5

3.0

2.5

2.0

1.5

1.0

Log Colony Diameter

20 40 60 80 100 120 140

20 40 60 80 100 120 140

[Allyl Alcohol]:

● 0 mM
● 5 mM
● 15 mM
● 20 mM

FIGURE 3.—Dependence of growth rate upon allyl alcohol concentration at 19°C and 37°C for ADHI haploid mutant strains LTR1 and HTR1 on YEPD + Antimycin A agar medium with 2% ethanol.

TABLE 2

Median asymptotic clone diameter (micrometer ± se median) for haploid mutant ADHI strains LTR1 and HTR1 at 19° and 37° for three concentrations of allyl alcohol

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Mutant</th>
<th>Allyl alcohol concentration</th>
<th>10 mM</th>
<th>5 mM</th>
<th>2.5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>19°</td>
<td>37°</td>
<td>19°</td>
<td>37°</td>
</tr>
<tr>
<td>YEPD + 2% ethanol</td>
<td>LTR</td>
<td>&gt;2000</td>
<td>0</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
</tr>
<tr>
<td>YEPD</td>
<td>LTR</td>
<td>34 ± 5</td>
<td>0</td>
<td>88 ± 2</td>
<td>34 ± 2</td>
</tr>
<tr>
<td></td>
<td>HTR</td>
<td>0</td>
<td>27 ± 2</td>
<td>34 ± 2</td>
<td>115 ± 15</td>
</tr>
<tr>
<td>YEPD + 0.04%</td>
<td>LTR</td>
<td>35 ± 4</td>
<td>0</td>
<td>100 ± 6</td>
<td>50 ± 4</td>
</tr>
<tr>
<td></td>
<td>HTR</td>
<td>0</td>
<td>0</td>
<td>46 ± 5</td>
<td>105 ± 8</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>LTR</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>290 ± 24</td>
</tr>
<tr>
<td></td>
<td>HTR</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>290 ± 24</td>
</tr>
</tbody>
</table>

Media include 1 ppm Antimycin A. "Zero" entries indicate no growth of isolated cells.

The stimulation of mutant strain growth, especially LTR1, by acetaldehyde in the absence of allyl alcohol may appear surprising, considering that added acetaldehyde depresses the allyl alcohol resistance of both mutant and wild-type strains in which respiration is genetically or pharmacologically blocked (WILLS...
acetaldehyde + NADH ⇌ ethanol + NAD⁺,

in the direction of the formation of ethanol and NAD⁺, and since the major metabolic role of ADH in yeast is the regeneration of NAD⁺ in glycolysis (SOLS, GANCEDO and DELAFUENTE 1969), such results suggest that the availability of NAD⁺ in the cytoplasm may limit glycolytic flux even when a functional cytoplasmic ADH is present. The effects of added ethanol in enhancing and added acetaldehyde in decreasing, allyl alcohol resistance are consistent with earlier experiments conducted at 30° with other mutants (WILLS and PHELPS 1978; WILLS and MARTIN 1980). These experiments demonstrated that allyl alcohol resistance is correlated with the ratio of NADH to NAD⁺ in the cytoplasm, which can be perturbed by the addition of either substrate or by functional mutations in ADH1.

The ability to select for different phenotypic classes of allyl-alcohol-resistant ADH functional mutants makes this experimental system useful for exploring relationships between enzyme structure and function, and cellular metabolism. In recent years, site-directed mutagenesis has become a powerful tool for analyzing the physical and chemical nature of enzyme catalysis. Through recombinant DNA techniques, specific structural variants can be created experimentally to examine the effects of particular amino acid substitutions upon the functional phenotype of the enzyme (e.g., WILKINSON et al. 1984; FERSHT et al. 1985; VILLAFLANCA et al. 1983; PIELAK, MAUK and SMITH 1985). The yeast alcohol dehydrogenase system is a complementary approach in which subsets of phenotypes (e.g., temperature-dependent allyl alcohol resistance) due to amino acid substitutions in ADH1 can be used to investigate how enzyme structure and function are shaped by the requirements of specific cellular and metabolic environments of the investigator's choosing.

Numerous comparative biochemical investigations of enzyme homologues strongly support the contention that fine-scale evolutionary adaptations at the level of enzyme function may occur with respect to not only temperature but also hydrostatic pressure, osmotic pressure and solute composition (HOCHACHKA and SOMERO 1984; SOMERO 1978; YANCY et al. 1982; SOMERO and BOWLUS 1983; SIEBENALLER and SOMERO 1978). The advantage of the present yeast ADH system is that it allows the investigation of the effects of single amino acid substitutions with the desired phenotypic effects, which is not possible in most comparative studies, where homologues may differ by numerous amino acid residues.

We thank T. MARTIN for technical assistance. This work was supported by National Institutes of Health grant GM32025 and a United States Public Health Service postdoctoral fellowship (J.G.H.).

LITERATURE CITED


