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Escherichia coli Mutants with a Temperature-Sensitive Alcohol Dehydrogenase

WILLIAM LOROWITZ AND DAVID CLARK*†

Department of Microbiology, University of Illinois, Urbana, Illinois 61801

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Mutants of *Escherichia coli* resistant to allyl alcohol were selected. Such mutants were found to lack alcohol dehydrogenase. In addition, mutants with temperature-sensitive alcohol dehydrogenase activity were obtained. These mutations, designated *adhE*, are all located at the previously described *adh* regulatory locus. Most *adhE* mutants were also defective in acetaldehyde dehydrogenase activity.

Ethanol is a major fermentation product of *Escherichia coli* (8). Acetyl coenzyme A (CoA) is converted to acetaldehyde by a CoA-linked acetaldehyde dehydrogenase, and the acetaldehyde is then reduced to ethanol by an NAD-coupled alcohol dehydrogenase. These two enzymes are expressed only anaerobically in wild-type cells. However, we have recently described derepressed mutants (*adhC*) in which both enzymes are produced during aerobic growth (6, 7). To continue our genetic analysis of this system, it was necessary to identify the structural genes for the two enzymes of the pathway. We have used a "suicide substrate," allyl alcohol, to isolate mutants lacking alcohol dehydrogenase. Aldehydes in which the keto group is conjugated with a double or triple bond are potent protein-alkylating agents. When the corresponding alcohols are incubated with alcohol dehydrogenase, they are converted to alkylating aldehydes (9, 10). Although the alcohol dehydrogenase is itself ultimately inactivated by alkylation of active site residues, most molecules of aldehyde escape from their site of formation and proceed to alkylate nearby enzyme molecules (1, 9). Hence, cells showing alcohol dehydrogenase activity may be selectively killed by treatment with the appropriate alcohols (which are themselves relatively innocuous). Butynyl ($CH_3C{\equiv}CCH_2OH$), propargyl ($CH{\equiv}CCH_2OH$), and allyl ($CH{=}CHCH_2OH$) alcohols are effective suicide substrates for alcohol dehydrogenase (1, 9, 10), and yeast mutants resistant to allyl alcohol have been shown to lack alcohol dehydrogenase (4, 5). Propargyl and allyl alcohols proved effective against *E. coli*, and we used the latter to isolate mutants lacking alcohol dehydrogenase or having a temperature-sensitive enzyme.

† Present address: Department of Microbiology, Southern Illinois University, Carbondale, IL 62901.

The *adhC* regulatory mutants, together with most of the genetic techniques and media, have been previously described (6). The ability of bacterial colonies to oxidize ethanol aerobically was observed by using tetrazolium redox-indicator plates (3) containing 0.5% ethanol. All bacteria were strains of *E. coli* K-12 and are listed in Table 1.

Alcohol dehydrogenase and acetaldehyde CoA dehydrogenase were assayed spectrophotometrically by following the reduction of NAD, using the high-speed supernatant fraction prepared from bacteria disrupted by passage through a French press at 10,000 lb/in^2 (6, 7). A unit of enzyme activity is defined as a nanomole of product formed per minute of incubation at 22°C. Inclusion of 4 mM dithiothreitol in extracts containing acetaldehyde CoA dehydrogenase greatly improved the stability of this enzyme. Thus, specific activities for acetaldehyde CoA dehydrogenase were some fivefold greater than those previously reported (7). The stability of alcohol dehydrogenase was not affected by dithiothreitol.

The wild-type strain DC271 was unaffected by 200 mM allyl alcohol when grown aerobically. However, when grown anaerobically, it was killed by 50 mM allyl alcohol. The *adhC*-derepressed strain DC272 was killed both anaerobically and aerobically, although 150 mM allyl alcohol was necessary in the latter instance. Thus, susceptibility to allyl alcohol correlated well with expression of alcohol dehydrogenase. Propargyl alcohol gave analogous results. Spontaneous mutants resistant to allyl alcohol were obtained by plating 10^7 to 10^8 cells onto rich broth plates containing 200 mM allyl alcohol (aerobic incubation; strain DC272 *adhC*) or onto rich broth-glucose plates with 100 mM allyl alcohol (anaerobic incubation; either wild-type or *adhC* strains). Allyl alcohol-resistant (AAR) isolates from strain DC272 (*adhC*) were streaked

TABLE 1. Bacterial strains

Strain	Relevant marker(s)[a]	Source or reference
DC271	*fadR*	(6)
DC272	*fadR adhC81*	(6)
DC300	*zch*::Tn*10 adhC81 fadR*	P1 (JW380) × DC272
DC343	Revertant of DC272	(6)
DC346	Revertant of DC272	(6)
JW380	*zch*::Tn*10* of W3110	J. Wechsler
WL1	*fadR adhC81 adhE1*	AAR mutant of DC272
WL2	*fadR adhC81 adhE2*	AAR mutant of DC272
WL3	*fadR adhC81 adhE3*	AAR mutant of DC272
WL33	*fadR adhC*$^+$ revertant	
WL64	*fadR adhC81 adhE14*(Ts)	Temperature-sensitive AAR mutant of DC272
WL66	*fadR adhC81 adhE16*(Ts)	Temperature-sensitive AAR mutant of DC272
WL68	*fadR adhC81 adhE18*(Ts)	Temperature-sensitive AAR mutant of DC272
WL69	*fadR adhC81 adhE19*(Ts)	Temperature-sensitive AAR mutant of DC272

[a] All DC and WL strains in this table are derivatives of *E. coli* Ymel and are F$^+$ *mel supF* and prototrophic. W3110 is a prototrophic strain with no other markers.

for single colonies on ethanol-tetrazolium indicator plates. Most formed white colonies, unlike colonies of the parent strain, which turned red. Almost all mutants forming white colonies on ethanol-tetrazolium plates had greatly reduced levels of alcohol dehydrogenase, whether they were selected under aerobic or anaerobic conditions.

TABLE 2. Enzyme activities of AAR mutants

Strain	Sp act (U/mg of protein) of enzyme[a]:			
	Aerobic		Anaerobic	
	ADH	ACDH	ADH	ACDH
DC271	<0.1	17.5	4.6	55
DC272	145	148	57	177
WL1	0.16	1.6	<0.1	1.5
WL2	<0.1	1.5	<0.1	2.2
WL3	<0.1	1.3	<0.1	1.6
WL33	0.14	12.0	8.6	42
DC343	1.24	0.5	<0.1	12.9
DC346	1.16	0.9	43	6.5

[a] Cells were grown aerobically on rich broth or anaerobically on rich broth plus Tris and gluconate. ADH, Alcohol dehydrogenase; ACDH, Acetaldehyde CoA dehydrogenase.

TABLE 3. Enzyme activities of temperature-sensitive mutants

Strain	Sp act (U/mg of protein) at following temp (°C) with indicated enzyme[a]:			
	30		42	
	ADH	ACDH	ADH	ACDH
DC272	115	101	175	148
WL64	31	41	21	24
WL66	78	162	81	328
WL68	8.4	5.0	16.4	8.7
WL69	1.7	51	<0.1	<0.1

[a] Cells were grown aerobically on rich broth at 30 or 42°C, and extracts were assayed at 22°C. ADH, Alcohol dehydrogenase; ACDH, Acetaldehyde CoA dehydrogenase.

The AAR mutants lacked both alcohol dehydrogenase and acetaldehyde CoA dehydrogenase. Enzyme activities are shown in Table 2 for the mutants WL1, WL2, and WL3, which showed almost total loss of enzyme activity both aerobically and anaerobically. Of some 30 AAR mutants assayed, none lacked alcohol dehydrogenase while retaining acetaldehyde CoA dehydrogenase. Mutant WL33 retained both enzymes under anaerobic conditions, yet lacked them when grown aerobically. It is thus probably an *adhC*$^+$ revertant. Table 2 also shows data for the mutants DC343 and DC346, previously isolated as revertants of strain DC272 *adhC* by a replica plating method (7). Both DC343 and DC346 proved to be resistant to allyl alcohol when tested. It is clear that the residual alcohol dehydrogenase activity in these strains in higher than those of WL1, WL2, and WL3.

To identify the structural gene for alcohol dehydrogenase, we isolated temperature-sensitive mutants resistant to allyl alcohol. An exponentially growing culture of strain DC272 in rich broth was treated with 2% ethyl methane sulfonate for 30 min at 37°C. After washing, the cells were suspended in broth and grown for several hours to allow segregation of the mutation and then plated onto agar containing allyl alcohol (50 or 100 mM) and incubated anaerobically at 42°C. Survivors were checked for allyl alcohol resistance and tested on ethanol indicator plates at 30 and 42°C. Isolates giving red colonies at 30°C and white colonies at 42°C were kept. The temperature-sensitive mutants fell into two classes. Class I, exemplified by strains WL64 and WL66, retained substantial amounts of enzyme activity in cells grown at 30 or 42°C (Table 3). However, the alcohol dehydrogenase from strains WL64 and WL66 grown at 30°C was more thermolabile (Fig. 1). Wild-type enzyme retained approximately 60% of its activity after 5 min at 50°C, whereas enzyme from WL64 and

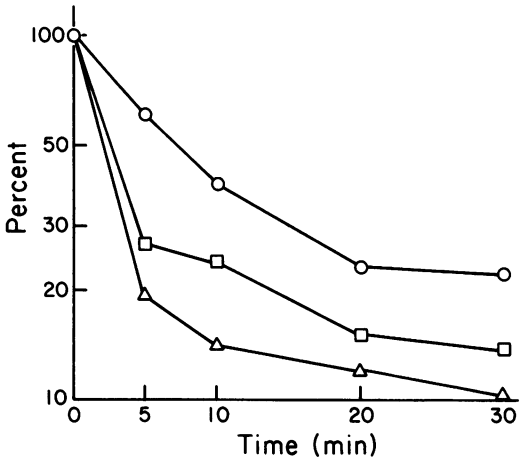

FIG. 1. Thermolability of alcohol dehydrogenase. Enzyme preparations were diluted 10-fold into potassium phosphate (50 mM, pH 7.4) containing 4 mM dithiothreitol. Samples were heated at 50°C, and samples were withdrawn at appropriate intervals for assay. Results are expressed relative to the unheated extracts. ○, strain DC272; □, strain WL66; △, strain WL64.

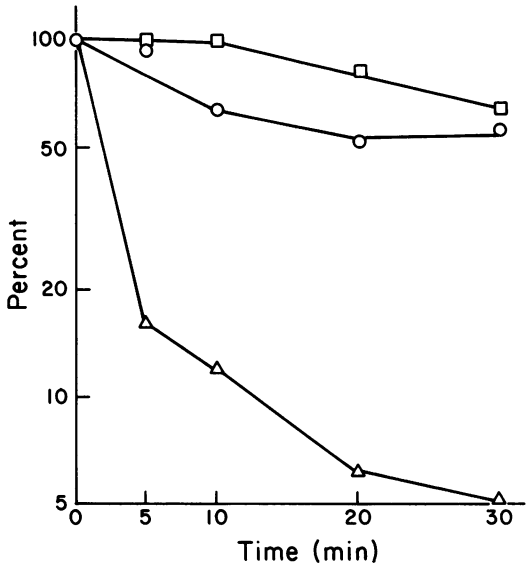

FIG. 2. Thermolability of acetaldehyde dehydrogenase. Extracts of strains DC272 (○), WL64 (△), and WL66 (□) were incubated at 50°C as described in the legend to Fig. 1 and assayed for enzyme activity at the times indicated. Results are expressed relative to the unheated extracts.

WL66 retained only approximately 20% activity.

The acetaldehyde CoA dehydrogenase activity in extracts prepared from strain WL64 was also found to be thermolabile, with similar inactivation kinetics to the alcohol dehydrogenase activity (Fig. 2). However, the acetaldehyde CoA dehydrogenase activity in extracts of strain WL66 was not altered. Further evidence for structural alteration of the alcohol dehydrogenase from strain WL64 was an altered pH profile (Fig. 3). The alcohol dehydrogenase activity from both the parental strain, DC272, and the mutant WL66 showed a pH optimum, with the activity at pH 10.5 almost equal to that at pH 9.5.

Class II temperature-sensitive mutants, exemplified by strains WL68 and WL69, had little detectable enzyme at either 30 or 42°C. We presume that the enzymes in such mutants are unstable in vitro, although they must be sufficiently stable in vivo at 30°C to allow the cells to grow on ethanol.

The AAR mutations were mapped by using appropriate insertions of Tn*10*. The *zch*::Tn*10* insertion in strain JW380 was found to be cotransducible with the *adhC* mutation in strain DC272. We therefore used contransducibility with *zch*::Tn*10* to map the various mutants described above (Table 4). The *adhE* mutations in strains WL1, WL2, WL3, DC343, and DC346; the *adhC*$^+$ revertant WL33; and the temperature-sensitive mutants WL64, WL66, WL68, and WL69 all cotransduced approximately 95%

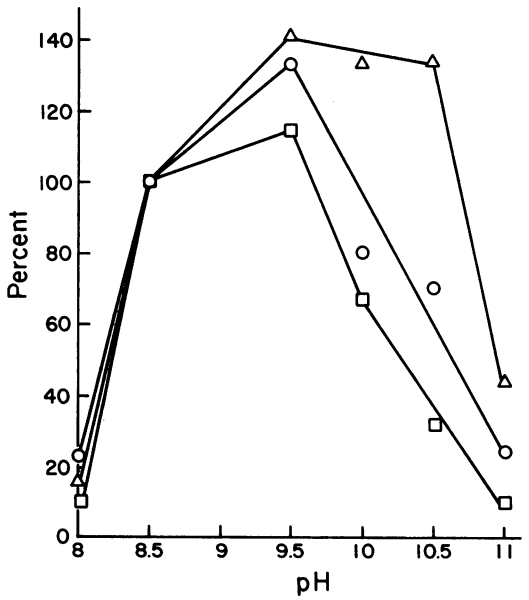

FIG. 3. pH Profile of alcohol dehydrogenase. Extracts of strains DC272 (○), WL64 (△), and WL66 (□) were assayed for alcohol dehydrogenase activity over a range of pH values. Sodium pyrophosphate (12 mM, pH 8.5) or the dipolar ionic buffers CHES [2(*N*-cyclohexylamino)ethane sulfonate] or CAPS [3(*N*-cyclocohexylamino)propane sulfonate] (50 mM, various pH values) were used. The results are expressed relative to the value for 12 mM pyrophosphate buffer, pH 8.5.

TABLE 4. Cotransduction experiments

P1 donor	Recipient	Marker selected	No. of colonies scored	% Cotransduction
JW380 *tet*	DC272 *adhC*[a]	*tet*	148	70
JW380 *tet*	DLM1 *chlC*	*tet*	100	67
JW380 *tet*	JC1552 *trp*	*tet*	100	51
JW380 *tet*	JC1552 *trp*	*trp*$^+$	100	40
DC300 *tet adhC*	WL1 *adhE*	*tet*	100	97
DC300 *tet adhC*	WL2 *adhE*	*tet*	90	97
DC300 *tet adhC*	WL3 *adhE*	*tet*	100	97
DC300 *tet adhC*	WL33 *adhC*$^+$	*tet*	100	91
DC300 *tet adhC*	DC271 *adhC*$^+$	*tet*	82	94
DC300 *tet adhC*	DC343 *adhE*	*tet*	100	95
DC300 *tet adhC*	DC346 *adhE*	*tet*	100	95
DC300 *tet adhC*	WL64 *adhE*(Ts)	*tet*	100	96
DC300 *tet adhC*	WL66 *adhE*(Ts)	*tet*	100	97
DC300 *tet adhC*	WL68 *adhE*(Ts)	*tet*	100	97
DC300 *tet adhC*	WL69 *adhE*(Ts)	*tet*	100	96

[a] The *adh* mutation was scored with tetrazolium-ethanol indicator plates, as detailed in the text.

with the *zch*::Tn*10* marker. Tetracycline-resistant derivatives of all of these strains which retained the *adhE* (or *adhC*$^+$) mutations were then used as P1 donors in a series of crosses with strain DC272 *adhC* as the recipient. The cotransduction frequencies of *adhE* with *zch*::Tn*10* in this reverse series of crosses were not significantly different from those shown in Table 4 (data not shown). All of these mutations are, therefore, located very close to the *adhC* locus (2), which defines a *cis*-dominant control site responsible for the aerobic repression of the two enzymes of the alcohol pathway, alcohol dehydrogenase and acetaldehyde CoA dehydrogenase (6, 7). The *adh* locus must also contain the structural gene for alcohol dehydrogenase, as is shown by our isolation of temperature-sensitive mutants.

Most mutants lacking alcohol dehydrogenase also showed greatly reduced levels of acetaldehyde CoA dehydrogenase. Furthermore, in strain WL64, both enzymes were temperature sensitive, suggesting that the presence of the alcohol dehydrogenase protein might be required for acetaldehyde CoA dehydrogenase activity. Other authors have suggested that the alcohol and acetaldehyde dehydrogenases of *E. coli* may be associated in some way (11, 12). Work is currently in progress to purify these enzymes and analyze possible structural interactions.

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