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The use of suicide substrates to select mutants of *Escherichia coli* lacking enzymes of alcohol fermentation

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Summary. Mutants of *Escherichia coli* resistant to chloroethanol or to chloroacetaldehyde were selected. Such mutants were found to lack the fermentative coenzyme A (CoA) linked acetaldehyde dehydrogenase activity. Most also lacked the associated fermentative enzyme alcohol dehydrogenase. Both types of mutants, those lacking acetaldehyde dehydrogenase alone or lacking both enzymes, mapped close to the regulatory *adhC* gene at 27 min on the *E. coli* genetic map. The previously described *acd* mutants which lack acetaldehyde dehydrogenase and which map at 63 min were shown to be pleiotropic, affecting respiration and growth on a variety of substrates. It therefore seems likely that the structural genes for both the acetaldehyde and alcohol dehydrogenases lie in the *adhCE* operon. This interpretation was confirmed by the isolation of temperature sensitive chloracetaldehyde-resistant mutants, some of which produced thermolabile acetaldehyde dehydrogenase and alcohol dehydrogenase and were also found to map at the *adh* locus. Reversion analysis indicated that mutants lacking one or both enzymes carried single mutations. The gene order in the *adh* region was determined by three point crosses to be *trp* – *zch*::Tn*10* – *adh* – *galU* – *bglY* – *tyrT* – *chlC*.

Key words: Acetaldehyde dehydrogenase – Alcohol dehydrogenase – Chloracetaldehyde – Anaerobic growth – Ethanol

Introduction

In *Escherichia coli* the changeover from aerobic to anaerobic growth is accompanied by the induction of up to 50 anaerobically regulated genes (Clark 1984) and the corresponding appearance of several new proteins (Smith and Neidhart 1983). Under anaerobic conditions the production of ethanol from fermentable sugars by *Escherichia coli* involves the conversion of acetyl-CoA to acetaldehyde by a coenyzme A-linked acetaldehyde dehydrogenase (ACDH) and the subsequent conversion of the acetaldehyde to ethanol by alcohol dehydrogenase (ADH) (Sokatch 1969: Dawes and Foster 1956). Both of these enzymes are coordinately induced under anaerobic conditions (Clark and Cronan 1980a, 1980b and regulatory mutations resulting in constitutive expression of both enzymes under aerobic conditions have been isolated and mapped to the *adh* locus at 27 min (Bachmann 1983; Clark and Cronan 1980a). The original regulatory mutations have been designated *adhC* in order to distinguish them from *adhE* mutations which also map in the *adh* locus but result in the loss of ADH enzyme activity (Lorowitz and Clark 1982). In an attempt to isolate mutants lacking ACDH, we previously used a replica plating technique to screen for colonies unable to interconvert ethanol and acetyl-CoA (Clark and Cronan 1980b). A mutant lacking ACDH but retaining ADH was isolated and the genetic lesion was found to map far from *adh* at a new locus designated *acd* (Clark and Cronan 1980b). Reinvestigation of the *acd* mutation indicated that this confers pleiotropic growth defects, as described below, and hence was not the structural gene for ACDH.

We have therefore devised a direct selection procedure for mutants lacking ACDH. In this paper we describe the use of 2-chloracetaldehyde which specifically kills cells expressing acetaldehyde dehydrogenase, presumably due to the lethal synthesis of chloroacetyl-CoA. Mutants selected by this procedure lacked ACDH and many also lacked ADH. Unlike the *acd* mutant these new ACDH mutants showed no pleiotropic growth defects and were found to map at the *adh* locus. In addition we selected mutants resistant to 2-chlorethanol, and found that most of these mutants were defective in both ADH and ACDH. Again these mutants were located at *adh*. Finally, we carried out three point crosses between several loci in the 27 minute region of the *E. coli* genetic map whose order was previously ambiguous. The genetic map of Bachmann (1983) needs to be slightly modified as follows: *trp* – *zch*::Tn*10* – *adh* – *galU* – *bglY* – *tyrT* – *chlC*.

Materials and methods

Bacterial strains and media. All bacteria were strains of *Escherichia coli* K12 and are listed in Table 1. Rich broth contained (per liter) tryptone (10 g), NaCl (5 g) and yeast extract (1 g). Minimal medium M9 (Miller 1972) was supplemented with carbon sources at 0.4% (w/v) and where appropriate with amino acids (50 mg/l). Metabolism of ethanol was screened using the tetrazolium indicator plates of Bochner and Savageau (1977) as modified previously (Clark and Cronan 1980b) and containing 0.5% (v/v) ethanol. Transductions were performed essentially as described by Miller (1972) and transductants were selected on medium E (Vogel and Bonner 1956) with glucose (0.4% w/v) or ethanol (0.5%) as carbon source. Tetracycline was used

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Table 1. Bacterial strains

Strain	Relevant markers	Source or Reference
CA10	*galU95 relA1 spoTl*	B. Bachmann
DC271	*fadR mel tyrT*	Lorowitz and Clark 1982
DC272	*fadR adhC81 mel tyrT*	Lorowitz and Clark 1982
DC300	*fadR mel tyrT adhC81 zch*::Tn*10*	Lorowitz and Clark 1982
DC349	*fadR mel tyrT adhC81 acd*	Clark and Cronan 1980b
DC356	*fadR mel tyrT adhC81 zgb*::Tn*10*	Clark and Cronan 1980b
DC637	*fadR mel tyrT adhC81 chlC*::Tn*10*	P1(RK5266) × DC272
ID18	*bglY206 thi-l tyrA2 cysB purC50 rpsL125*	M. De Felice
JM246	*cysI53*(Am)	B. Bachmann
JW380	*zch*::Tn*10*	J. Wechsler
PRC66	*zch*::Tn*10 adhC81 bglY*	P1(DC300) × ID18
PRC70	*zch*::Tn*10 adhC81 galU*	P1(DC300) × CA10
PRC79	*zch*::Tn*10 adhC81 bglY*	P1(PRC66) × W1485
PRC95	*trpE63* of MC4100	Laboratory collection
PRC111	*zch*::Tn*10 adhC81 bglY cysI53*(Am)	P1(PRC66) × JM246
PRC114	*zch*::Tn*10 adhC81 galU bglY cysI53*(Am)	P1(PRC70) × JM246
PRC122	*zch*::Tn*10 tyrT*	P1(DC271) × PRC114
PRC124	*zch*::Tn*10 adhC81 galU tyrT cysI53*(Am)	P1(PRC122) × PRC124
PRC131	*zch*::Tn*10 bglY cysI53*(Am)	P1(PRC122) × PRC111
PRC301 to 343	chloroethanol-resistant mutants of DC272	See text
PRC401 to 481	chloroacetaldehyde-resistant mutants of DC272	See text
PRC500	*zch*::Tn*10* of PRC436	P1(DC300) × PRC436
PRC506 PRC515 PRC517 PRC520	temperature-sensitive chloroacetaldehyde-resistant mutants of DC272	See text
PRC607	*chlC*::Tn*10* of DC272	P1(DC637) × DC272
PRC627	*chlC*::Tn*10* of PRC436	P1(PRC607) × PRC436
PRC629	*zch*::Tn*10* of DC271	P1(DC300) × DC271
PRC633	*chlC*::Tn*10* of WL1	P1(PRC607) × WL1
RK5266	*chlC*::Tn*10*	V. Stewart
W1485	wild type	B. Bachmann
MC4100	*araD139* DE(*argF – lac*) *rpsL150 thiA ptsF25 relA1*	M. Casadaban
WL1	*fadR mel tyrT adhC81 adhE1*	Lorowitz and Clark 1982

at 10 mg/l and neomycin sulfate at 5 mg/l. Media were solidified with 1.5% (w/v) agar. Chloroacetaldehyde (8 mg/l) and chlorethanol (5 mg/ml) were used in minimal succinate media for selecting resistant mutants. Anaerobic growth was performed in Oxoid anaerobic jars under a H_2/CO_2 atmosphere generated by means of Oxoid Gas Generating Kits. All media used for anaerobic growth were supplemented with trace elements as follows: $FeSO_4$ (50 μM) H_2SeO_3 (5 μM) and $(NH_4)_6Mo_7O_{24}$ (5 μM with respect to Mo).

Materials. Coenzyme A (lithium salt) was from P.L. Biochemicals, chloroacetaldehyde from Fluka, allyl alcohol and chloroethanol from Aldrich, MOPS and CHES buffers from Research Organics, and antibiotics, nicotinanide adenine dinucleotide (NAD), and other biochemicals were from Sigma.

Isolation of temperature-sensitive mutants. We used chloroacetaldehyde to select mutants which have a temperature sensitive ACDH. To do this, DC272 was grown to exponential phase in M9-succinate and then treated with 1% ethyl methane sulfonate for 30 min at 30° C. The cells were then washed twice and resuspended in M9-succinate at 30° C. After several hours the culture was diluted tenfold, placed at 42° C for 1 h and chloroacetaldehyde (10 mg/l) was added. When the chloroacetaldehyde-treated cells reached stationary phase, they were washed twice, diluted tenfold and resuspended in M9-ethanol. Following overnight growth at 30° C the cells were washed twice, resuspended in M9-succinate and 0.1 ml samples were plated on M9-succinate + 8 mg/l chloroacetaldehyde and the plates incubated at 42° C. From these plates colonies were spotted onto ethanol indicator plates and those colonies which were red at 30° C and white at 42° C were kept.

Enzyme assays. Bacteria were grown in 200 ml batches to approximately 10^9 /ml in rich broth and harvested by centrifugation. After washing in MOPS [3-(N-morpholino)–propanesulfonate] buffer (50 mM, pH 7.4) the cell pellet was resuspended in 2.5 ml of MOPS buffer containing 4 mM dithiothreitol 10 mM $MgSO_4$ and 10 μM $MnSO_4$ and the cells were broken by passage through a French pressure cell (Aminco) at 20,000 psi. The high speed supernatant fraction was obtained by centrifugation at 145,000 *g* for 60 min and was used for enzyme assay.

Both alcohol and acetaldehyde dehydrogenase were assayed spectrophotometrically by following the reduction of NAD^+ to NADH at 340 nm. For ADH the assay mix contained NAD^+ (75 nmol), ethanol (20 μl), enzyme preparation (5–50 μl), and 12 mM sodium pyrophosphate (pH 8.5) to give a final volume of 1.0 ml. For ACDH the assay was performed in 50 mM CHES [2-(N-cyclohexylamino)-ethanesulfonate] buffer (pH 9.5) containing 1 mM dithiothreitol. The 1.0 ml assay volume contained NAD^+ (75 nmol), lithium coenzyme A (100 nmol), enzyme preparation (5–50 μl) and the reaction was started by adding 10 μl of 1.0 M acetaldehyde. One unit of enzyme activity is defined as one nmole of NADH formed per minute.

Results

The acd mutation causes pleiotropic growth defects

Our original acetaldehyde dehydrogenase mutant, DC349 (*acd*) (Clark and Cronan 1980b) was found to show pleiotropic growth defects. As shown in Table 2 DC349 was unable to grow on most carbon sources other than fermentable sugars, suggesting some deficiency in respiratory metabolism. In support of this it was found that DC349 is

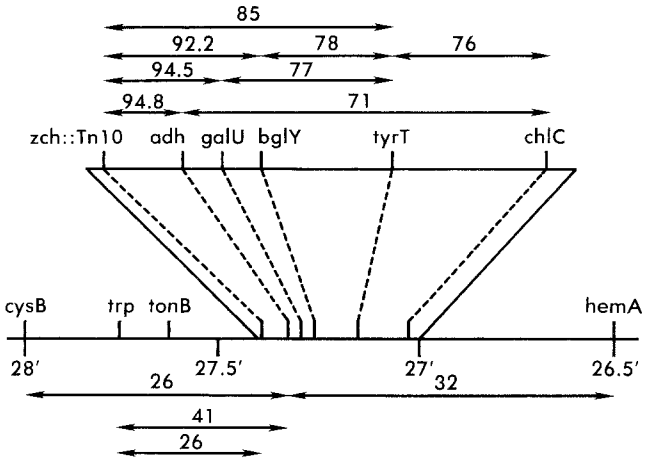

Fig. 1. Genetic map of the *adh* region. Data are from this work and from Clark and Cronan (1980a, 1980b) and Lorowitz and Clark (1982). The linkage data are average cotransduction frequencies from several crosses

resistant to neomycin, an antibiotic whose uptake depends on respiratory function (Ball and Chopra 1982), whereas the parent strain is neomycin sensitive. These observations led us to isolate more ACDH negative mutants in the hope of obtaining some without such additional defects.

Selection of chloroacetaldehyde-resistant mutants

Preliminary observations showed that chloroacetaldehyde was a good substrate for ACDH (data not shown) and that it was selectively toxic to strains expressing this enzyme. Wild-type *E. coli* express ACDH anaerobically but not aerobically, whereas *adhC* mutants express ACDH under both conditions (Clark and Cronan 1980b). It was found that *adhC* strains were hypersensitive to chloroacetaldehyde both aerobically and anaerobically, whereas wild type strains were relatively resistant when grown aerobically.

Chloroacetaldehyde-resistant mutants were isolated from DC272 *adhC*. After overnight growth in M9-succinate medium, aliquots of approximately 10^7 cells were spread on plates of minimal succinate medium containing 8 mg/l of chloroacetaldehyde, a concentration which was lethal to DC272. Resistant colonies were re-purified on ethanol indicator plates, and white colonies were picked. Strains expressing both ACDH and ADH give a red reaction on this indicator medium whereas strains lacking either enzyme form white colonies. Seventy independently isolated, chloroacetaldehyde-resistant mutants were picked and tested on the carbon sources shown in Table 2. All of the mutants isolated by chloroacetaldehyde resistance grew on all of these carbon sources except for ethanol.

Mapping of chloroacetaldehyde-resistant mutants.

Strain DC300 contains an insertion of Tn10 which is approximately 95% cotransducible with *adh* (Fig. 1), whereas the Tn10 insertion in DC356 is about 30% cotransducible with the *acd* locus. P1 *vir* grown on these strains was crossed with the chloroacetaldehyde-resistant mutants and tetracycline-resistant transductants were tested on ethanol indicator plates. All of the chloroacetaldehyde-resistant mutants showed >90% cotransduction with the *zch*::Tn*10* insertion which lies close to *adh* (Table 3). No cotransduction was observed with *zgb*::Tn*10* which is near *acd*.

Table 2. Growth defects of *acd* mutant[a]

Carbon source[b]	Strain		
	DC271 (*adh*$^+$)	DC272 (*adhC*)	DC349 (*adhC acd*)
Ethanol	−	+	−
Acetate	+	+	−
Succinate	+	+	−
Lactate	+	+	−
Glycerol	+	+	±
Glucose	+	+	+
Gluconate	+	+	+
Xylose	+	+	±
Mannitol	+	+	±

[a] + indicates full growth, ± indicates slow growth, and − indicates no growth

[b] Growth tests were performed in M9-minimal medium with 0.4% w/v carbon source except for ethanol (0.5% v/v)

Table 3. Cotransductional mapping of chloroacetaldehyde resistant mutants[a]

P1 donor	Recipient	Colonies scored	% Cotransduction
DC300 *zch*::Tn*10*	PRC436	450	97
DC300 *zch*::Tn*10*	PRC438	78	97
DC300 *zch*::Tn*10*	PRC439	68	97
DC300 *zch*::Tn*10*	PRC465	75	97
DC300 *zch*::Tn*10*	PRC480	48	96
DC300 *zch*::Tn*10*	PRC481	66	95
DC300 *zch*::Tn*10*	PRC506	200	98
DC300 *zch*::Tn*10*	PRC515	50	96
DC300 *zch*::Tn*10*	PRC517	78	97
DC300 *zch*::Tn*10*	PRC520	50	92
DC356 *zgb*::Tn*10*	PRC436	150	0
DC637 *chlC*::Tn*10*	DC272	100	50
PRC500 *adhE436 zch*::Tn*10*	DC272	100	93
PRC607 *chlC*::Tn*10*	PRC436	600	40

[a] The selected marker in all crosses was tetracycline resistance. The PRC strains used as recipients are all chloroacetaldehyde-resistant mutants of DC272

Enzyme assays

The alcohol dehydrogenase and acetaldehyde CoA dehydrogenase levels were both assayed in each of the seventy chloroacetaldehyde-resistant mutants, as described in Materials and methods. All of the mutants had less than 5% of the parental level of ACDH (Table 4). The mutants fell into three classes based on levels of ADH. The most frequent type of mutant (Class I) had very little ADH activity, i.e., <10% of parental. Of the 70 mutants tested 55 were defective in both enzymes. Class II mutants retained partial ADH activity, i.e., up to 50% of the parental level, and comprised ten mutants. Those mutants retaining essentially full ADH activity constitute Class III; there were only five of these.

Table 4. Enzyme levels in chloroacetaldehyde-resistant mutants[a]

Strain	Specific activity (units/mg protein)	
	ADH	ACDH
DC271 *adh*$^+$	0.1	1.6
DC272 *adhC*	85	115
Class I		
PRC438	0.6	0.6
PRC439	0.5	0.5
Class II		
PRC465	15.9	1.3
PRC480	31.7	0.6
Class III		
PRC436	84.4	0.6
PRC481	69.2	0.4

[a] ADH and ACDH were assayed in a series of chloroacetaldehyde-resistant mutants. Representative assays are given for strains from each of the three mutant classes. Cultures were grown aerobically in rich broth

Table 5. Chloroethanol resistant mutants

Strain	MIC to CA[a]	Enzyme level (%)[b]	
		ADH	ACDH
DC271 *adh*$^+$	16	1.0	1.8
DC272 *adhC*	2	100	100
Class I[c]			
PRC318	8	1.4	5.2
PRC322	16	1.4	2.5
PRC322	16	1.4	2.6
Class II[c]			
PRC327	8	30.0	14.0
Class III[c]			
PRC304	16	146	3.8
PRC340	16	173	1.9
PRC342	16	109	3.4

[a] Minimal inhibitory concentration to chloroacetaldehyde in micrograms per ml in succinate minimal medium

[b] Enzyme levels are shown relative to DC272. Cultures were grown aerobically in rich broth

[c] The mutants are divided into classes equivalent to those for the chloroacetaldehyde-resistant mutants (see Table 4)

Chloroethanol-resistant mutants

Chloroethanol also proved to be selectively toxic to *E. coli* strains which express ADH and ACDH. Mutants of DC272 (*adhC*) were selected for resistance to chloroethanol (5 mg/ml). Some proved to be defective in both ADH and ACDH, whereas others retained ADH activity (Table 5). The chloroethanol mutants were found to be resistant to chloroacetaldehyde and, not surprisingly, the extent of chloroacetaldehyde resistance correlated with the residual level of ACDH enzyme activity (Table 5). These mutants also mapped at the *adh* locus (data not shown).

Some of the chloroethanol resistant mutants of Class III had lost ACDH activity yet showed ADH activity greater than the parental strain, DC272 (Table 5). As discussed below, it is likely that ACDH and ADH form some sort of bifunctional complex. It is thus possible that the mutations of PRC304 and PRC340 affect the structure of this complex in such a way as to increase one of its activities (ADH) and yet largely eliminate the other (ACDH).

Temperature-sensitive mutants

The above data suggest that the structural gene for ACDH is in the ADH region instead of at the *acd* locus. We therefore used chloroacetaldehyde to select several mutants which were temperature sensitive (see Material and methods). Most of these mutants, e.g., PRC515 and PRC520 (Table 6), had little detectable ADH or ACDH at 30° C or 42° C. This is presumably because the enzymes are unstable in vitro even through they were sufficiently stable at 30° C in vivo to allow the formation of red colonies on ethanol indicator plates. A few of the temperature-sensitive mutants did contain substantial amounts of enzyme when grown at the lower temperature, though little activity was found at 42° C. Using one of these strains, PRC517, we were able to demonstrate the thermolability of both ACDH and ADH (Fig. 2). Both enzyme activities in extracts of PRC517 grown at 30° C were completely inactivated after 5 min at 50° C whereas such treatment had little effect on wild-type enzyme from DC272. The mutations in these strains were mapped by cotransduction with the *zch*::Tn*10* in DC300 (Table 3) and found to be >90% cotransducible with this marker.

Anaerobic growth of mutants

We have surveyed the growth properties of representative strains under anaerobic conditions. Both wild type and *adhC* strains grew on minimal medium with fermentable sugars (glucose, mannose, fructose) and sugar derivatives (gluconate, sorbitol, mannitol) under both aerobic and anaerobic conditions. Mutants which lack either or both of ADH and ACDH were unable to grow anaerobically on these sugars or their derivatives. However, they were able to grow aerobically and could grow anaerobically if alternative electron acceptors, such as nitrate, were added.

Reversion analysis

Revertants of *adhE* mutants of various enzyme phenotypes were selected by two methods. The *adhC* parent of the mutants, DC272, can grow on ethanol as sole carbon and energy source, whereas mutants defective in either or both of ADH and ACDH cannot. Consequently revertants may be selected on M9-ethanol medium. Mutants defective in either or both of ADH and ACDH are also incapable of anaerobic growth on fermentable sugars such as glucose. Thus, revertants may also be selected for anaerobic growth on glucose-minimal medium. Revertants were selected from PRC436, PRC438 and PRC439 by both methods. Irrespective of the selection procedure all of the revertants analyzed (>100) were red on alcohol indicator medium, used ethanol as carbon source aerobically and were able to grow anaerobically on glucose as sole carbon and energy source. Representative revertants derived from each of PRC436,

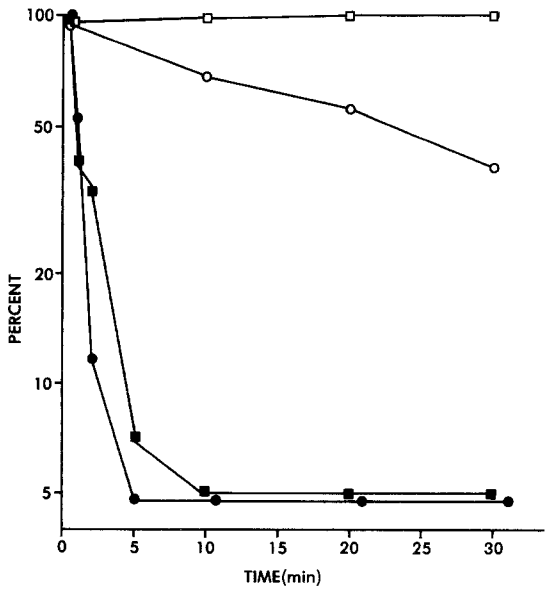

Fig. 2. Thermolability of ADH and ACDH from PRC517 and DC272. Samples of enzyme preparations were heated at 50° C and aliquots were withdrawn at the appropriate intervals for assay. Activity is expressed as percent activity of the unheated sample. The 100% values are those for cultures grown in rich broth at 30° C as shown in Table 6. □, strain DC272 (ADH); ○, strain DC272 (ACDH); ■, PRC517 (ADH); ●, PRC517 (ACDH)

Table 6. Enzyme activities of DC272 and temperature-sensitive mutants[a]

Strain	Specific activity (units/mg protein)			
	30° C		42° C	
	ADH	ACDH	ADH	ACDH
DC272	71	120	86	156
PRC506	37	95	35	63
PRC515	5	3	5	3
PRC517	15	29	9	6
PRC520	1	3	2	3

[a] Cells were grown aerobically in rich broth at 30° C or 42° C as indicated and the enzyme preparations were assayed at 22° C. ADH, alcohol dehydrogenase; ACDH, acetaldehyde dehydrogenase

PRC438 and PRC439 were mapped. P1 grown on each revertant was crossed with PRC95 (*adh*$^+$ *trpE*) and *trp*$^+$ transductants were selected. Approximately 40% of these were able to use alcohol as carbon source (i.e., were *adhC*, *adhE*$^+$). This demonstrated that the reversion events were in the *adh* region, rather than being secondary mutations elsewhere in the chromosome. These selected revertants were also assayed for their enzyme levels. Values ranged from 75% to 150% of those observed in strain DC272 grown under the same conditions.

Mapping of genes near adh

Several of the genes in the *adh* region have never been ordered precisely. We, therefore, carried out a series of three point crosses in order to resolve these ambiguities. The results are shown in Table 7 and indicate a gene order of *trp*, *zch*::Tn*10*, *adh*, *galU*, *bglY*, *tyrT*, *chlC*. These and previous data are incorporated into Fig. 1. The presence of *tyrT* (*supF*) was scored either by suppression of the *cysI* (Am) mutation from JM246 or by sensitivity to T4(Am).

Discussion

Our previous work resulted in the isolation of *adhC* mutants which express the two anaerobic enzymes ADH and ACDH constitutively (Clark and Cronan, 1980a). Subsequently, we isolated mutants which lacked ACDH but retained ADH. These *acd* mutants mapped far away from the orginal *adh* locus (Clark and Cronan, 1980b). However, further investigation showed that the *acd* mutation was pleiotropic and interfered in some as yet uncharacterized way with respiratory metabolism. We, therefore, devised a direct suicide selection of mutants specifically lacking ACDH. This procedure resembles our previous selection of mutants lacking ADH (Lorowitz and Clark 1982). Both selections rely on lethal syntheses: the conversion of allyl alcohol to acrylaldehyde by ADH (Rando 1974) or of chloroacetaldehyde to chloroacetyl-CoA by ACDH. In addition, we used chlorethanol which is also converted to chloroacetyl-CoA but requires both ADH and ACDH to achieve this. Several acetyl-CoA using enzymes are known to be inactivated by halogenated alkyl-CoA derivatives (Owens and Barden 1978; Bloxham et al. 1978). It is uncertain which of these several vital enzymes is the in vivo target. In higher concentrations chloroacetaldehyde is generally toxic, probably due to direct chemical reaction with DNA (Hall et al. 1981; Kayasuga-Mikado et al. 1980). However in lower amounts, chloroacetaldehyde is selectively toxic to cells expressing ACDH activity. Starting with an *adhC* parent with constitutive expression of high levels of ACDH we found that all of approximately 70 chloroacetaldehyde-resistant mutants lacked ACDH activity and most lacked ADH also. Furthermore, selection of mutants resistant to chloroethanol gave similar results. These mutants all mapped at the *adhCE* locus and showed none of the growth defects found in the *acd* mutant, DC349. When we previously selected mutants lacking ADH directly, we found that almost all were also lacking ACDH (Lorowitz and Clark 1982). Mutants which were temperature-sensitive for chloroacetaldehyde resistance were found to possess ADH and ACDH which were both thermolabile in vitro. These observations support the idea of some sort of molecular association between ADH and ACDH. Previous work by Rudolph et al. (1968) indicated that purified ACDH is "contaminated" with some ADH activity. Furthermore Schmitt (1975) suggested that the ACDH activity of *E. coli* was in a particle of high molecular weight as a result of sedimentation experiments. We are presently purifying these enzymes and have found that both activities are associated with a protein of native molecular weight slightly greater than 200 kilodaltons.

Analysis on sodium dodecyl sulfate/polyacrylamide gels indicated subunits of approximately 110 kilodaltons (Koepke and Clark, manuscript in preparation). Such an association has been observed for the corresponding two enzymes from *Clostridium* which form a macromolecular complex (Lurz et al. 1979). At present, it is unclear whether two identical subunits each carrying both enzyme activities or two distinct proteins of very similar molecular weight

Table 7. Three-factor crosses

P1 Donor	Recipient	Selected Marker	Number of Recombinants			
			adh$^+$ *gal*$^+$	*adh*$^+$ *galU*	*adhC gal*$^+$	*adhC galU*
PRC70 *zch*::Tn*10 adhC galU*	W1485	Tet	9	1	9	181
PRC70 *zch*::Tn*10 adhC galU*	JM246	Tet	17	2	5	276
PRC79 *zch*::Tn*10 adhC*	CA10 *galU*	Tet	1	20	865	12
			adh$^+$ *bgl*$^+$	*adh*$^+$ *bglY*	*adhC bgl*$^+$	*adhC bglY*
DC300 *zch*::Tn*10 adhC*	ID18 *bglY*	Tet	1	22	271	6
PRC66 *zch*::Tn*10 adhC bglY*	W1485	Tet	16	1	8	155
PRC66 *zch*::Tn*10 adhC bglY*	JM246	Tet	3	0	2	95
			gal$^+$ *bgl*$^+$	*gal*$^+$ *bglY*	*galU bgl*$^+$	*galU bglY*
PRC79 *zch*::Tn*10 bglY*	CA10 *galU*	Tet	5	861	32	0
			adh$^+$ *tyrT*$^+$	*adh*$^+$ *tyrT*	*adhC tyrT*$^+$	*adhC tyrT*
DC300 *zch*::Tn*10 adhC tyrT*	JM246	Tet	15	0	13	148
PRC607 *adhC tyrT chlC*::Tn*10*	JM246	Tet	24	5	0	71
DC271 *tyrT*	PRC114 *adhC galU*	*gal*$^+$	4	482	4	10
DC300 *zch*::Tn*10 tyrT*	W1485	Tet	4	10	7	158
			gal$^+$ *tyrT*$^+$	*gal*$^+$ *tyrT*	*galU tyrT*$^+$	*galU tyrT*
PRC114 *zch*::Tn*10 galU*	PRC122 *tyrT*	Tet	0	27	445	28
			bgl$^+$ *tyrT*$^+$	*bgl*$^+$ *tyrT*	*bglY tyrT*$^+$	*bglY tyrT*
PRC111 *zch*::Tn*10 bglY*	PRC122 *tyrT*	Tet	7	54	395	44
			gal$^+$ *bgl*$^+$	*gal*$^+$ *bglY*	*galU bgl*$^+$	*galU bglY*
PRC124 *galU tyrT*	PRC131 *bglY*	*tyrT*[a]	9	108	383	0

[a] Both PRC124 and PRC131 carry the *cysI53* amber mutation, hence it is possible to select for transfer of *tyrT* by selecting Cys$^+$

constitute this complex. Depending on which of these alternatives turns out to be correct, the *acd* gene may either code for one the subunits (if dissimilar) or, if the subunits are identical, it may be involved in activation of the binding site for coenzyme A which is only required for ACDH activity. Although the great majority of mutants had lost both enzymes, a few strains lacking ACDH yet retaining almost parental levels of ADH were found. We assume that in these rare mutants the active site for ACDH is damaged yet the structural integrity of the enzyme complex is unharmed, allowing ADH to function.

Another unresolved question is why mutants blocked in the conversion of acetyl-CoA to ethanol cannot grow anaerobically by fermenting pyruvate to lactate. Guest (1979) has shown that mutants lacking phosphotransacetylase and/or acetate kinase, which are thus impaired in the conversion of acetyl-CoA to acetate, are also unable to grow anaerobically. In contrast mutants lacking pyruvate formate lyase (*pfl*) and thus unable to produce either ethanol or acetate, are capable of anaerobic growth on glucose provided they are supplemented with acetate for anabolic purposes. However, *pfl* mutants are unable to grow anaerobically on substrates more reduced than glucose (e.g., sugar alcohols such as sorbitol) even if supplemented with acetate (Clark, unpublished). Our working hypothesis is that the ability to grow anaerobically depends not only on the ability to produce fermentation products such as acetate, ethanol or lactate but also requires that the proportions of these products be adjusted to account exactly for the excess reducing equivalents present in the growth substrate. Thus, unbalancing the conversion of acetyl-CoA to an equal mixture of ethanol and acetate impairs anaerobic growth irrespective of whether the production of acetate or of ethanol is blocked. We are presently constructing strains with various combinations of mutations affecting ethanol production, acetate production and lactate production in order to test this hypothesis (Mat-Jan and Clark, unpublished).

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