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Escherichia coli Mutants with Altered Control of Alcohol Dehydrogenase and Nitrate Reductase

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Mutants of *Escherichia coli* which overproduce alcohol dehydrogenase were obtained by selection for the ability to use ethanol as an acetate source in a strain auxotrophic for acetate. A mutant having a 20-fold overproduction of alcohol dehydrogenase was able to use ethanol only to fulfill its acetate requirement, whereas two mutants with a 60-fold overproduction were able to use ethanol as a sole carbon source. The latter two mutants produced only 25% of the wild-type level of nitrate reductase, when grown under anaerobic conditions. Alcohol dehydrogenase production was largely unaffected by catabolite repression but was repressed by nitrate under both aerobic and anaerobic conditions. The genetic locus responsible for alcohol dehydrogenase overproduction was located at min 27 on the *E. coli* genetic map; the gene order, as determined by transduction, was *trp tonB adh chlC hemA*. The possible relationship of alcohol dehydrogenase to anaerobic redox systems such as formate-nitrate reductase is discussed.

During experiments in which certain fatty acid derivatives were fed to acetate auxotrophs, we inadvertently isolated mutants that were able to use, as an acetate source, the small amounts of ethanol in which the fatty acid derivatives had been dissolved.

Wild-type *Escherichia coli* strains are unable to use ethanol as a carbon source. However, they do produce ethanol as a fermentation product under certain conditions of anaerobic growth (17), and the presence of an alcohol dehydrogenase (ADH) has been reported (19). In the absence of nitrate, at acidic pH, and especially in the presence of high phosphate concentrations, *E. coli* ferments glucose mainly to lactic acid. However, at alkaline pH and in the absence of phosphate an equimolar mixture of acetate plus ethanol is the major product (17). We therefore investigated our mutants under a variety of growth conditions. We found that the utilization of ethanol by these strains can be attributed to overproduction of ADH and that the levels of this enzyme vary in response to growth conditions. We have mapped the genetic locus responsible for the phenotype of these mutants and found it to be very close to *chlC*, the structural gene for one of the subunits of nitrate reductase (NR) (3). This is particularly interesting, since the presence of nitrate suppresses production of ADH, and the level of NR is lower in certain of these mutants. The possible relationship between the various redox systems specific to anaerobic growth is considered.

MATERIALS AND METHODS

Bacterial strains. All bacteria were strains of *Escherichia coli* K-12 and are listed in Table 1. A series of strains containing the *aceF* and *fadR* mutations as well as a variety of markers in the 25- to 28-min region of the *E. coli* map were constructed for mapping purposes. Strain JC1552 (*leu*) was transduced to Leu$^+$ with P1 grown on strains A10 (*aceF10*) or KΔ15 (an *aceEF* deletion mutant), to give the *ace* derivatives DC21 (*leu$^+$ aceF10*) and DC34 (leu$^+$ Δ*ace-15*). Strains DC21 and DC34 were made *fadR* by selection for derivatives able to use decanoic acid as a carbon source (13). Strains DC33 (DC21 *fadR*) and DC38 (DC34 *fadR*) both retain the *trp* marker of strain JC1552. These strains were transduced to Trp$^+$ using P1 grown on a variety of donor strains to give *aceF fadR* strains containing the *pyrF*, *cysB*, and *hemA* mutations (see Table 1). Strain DC24 (*leu purB*) was constructed by crossing the Hfr strain KL333 (*leu lac$^+$*) with strain H680 (*purB lac rpsL*) and selecting for Str^r Lac$^+$. *aceF* and *fadR* derivatives of DC24 were made as described above. The *recA* strains DC340 and DC341 were constructed by transducing DC280 and DC281, respectively, to tetracycline resistance with P1 grown on strain NK6659 *recA srl*::Tn*10*. UV-sensitive transductants were picked and purified.

Media. Rich broth contained (per liter) tryptone (10 g), NaCl (5 g), and yeast extract (1 g) and was supplemented with 0.1% sodium acetate for *aceF* strains. Except when decanoic acid was the carbon source, minimal medium was medium E (20). Mixtures of decanoic acid and the citrate present in medium E are bactericidal (unpublished observations), and therefore, when using decanoic acid as a carbon source, we used M9 medium (13). Solid media contained 1.5% agar.

Minimal media were supplemented with L-amino acids (50 μg/ml), nucleotides (25 μg/ml), and thiamine (1 μg/ml) as appropriate. The carbon sources used were glucose (0.4%), succinate (0.4%), acetate (0.2%), decanoate (0.1% solubilized with 1% Brij 58), and ethanol (0.5%).

TABLE 1. *Bacterial strains*

Strain	Relevant markers	Source[a]
JC1552	*argG6 metB1 his-1 leu-6 trp-31 mtl-2 xyl-7 malA1 gal-6 lacY1 rpsL-104*	CGSC 4273
KL333	HfrC *leu-40 lacZ608*	CGSC 4337
H680	*thi-1 tyrA2 his-68 trp-45 purB51 lacY1 malA1 mtl-2 xyl-7 gal-6 rpsL125*	CGSC 5038
UB1005	*metB1*	J. B. Beard
SHSP19	*hemA8 metB1*	CGSC 4679
DLM1	*thi-1 chlC*	J. A. DeMoss
TS9A	*thi-1 chlC*(Ts)	J. A. DeMoss
AB3078	*thi-1 leu-6 thyA6 pyrF32*	CGSC 3078
F15/KL110	Donor of F15 $thyA^+$	K. B. Low
NH4104	Donor of F42 lac^+	CGSC 4349
CSH48	Carries FColV, B trp^+, ϕ80 lysogen	K. B. Low
F125/KL181	Donor of F125 trp^+ $purB^+$	CGSC 4320
DC24	*leu-40 tyrA2 his-68 thi-1 purB51* lac^+ Str^r	KL333 × H680
DC172	*cysB*	This laboratory
DC189	$purB^+$ *hemA* derivative of H680	P1(SHSP19) × H680
NK6659	*recA srl*::Tn*10*	N. Kleckner
aceF strains		
A10	*aceF10*	U. Henning
KΔ15	*thyA met* Δ(*aroP-aceF*)	J. R. Guest
DC25	As DC24 but leu^+ *aceF10*	P1(A10) × DC24
DC31	Spontaneous *fadR* of DC25	
DC35	As DC24 but leu^+ Δ*ace-15*	P1(KΔ15) × DC24
DC39	Spontaneous *fadR* of DC35	
DC21	As JC1552 but leu^+ *aceF10*	P1(A10) × JC1552
DC33	Spontaneous *fadR* of DC21	
DC34	As JC1552 but leu^+ Δ*ace-15*	P1(KΔ15) × JC1552
DC38	Spontaneous *fadR* of DC34	
DC68	trp^+ *pyrF* derivative of DC33	P1(AB3078) × DC33
DC69	trp^+ *pyrF* derivative of DC38	P1(AB3078) × DC38
DC185	trp^+ *hemA* derivative of DC33	P1(SHSP19) × DC33
DC186	trp^+ *cysB* derivative of DC33	P1(DC172) × DC33
DC244	Spontaneous *gyrA* of DC33	
DC248	$cysB^+$ *trp hemA* derivative of DC186	P1(DC189) × DC186
DC285	hem^+ *chlC* derivative of DC248	P1(DLM1) × DC248
DC47	Spontaneous *fadR* of A10	
DC48	Spontaneous *fadR* of A10	
DC80	*aceF10 fadR adh-80*	Ethanol-utilizing mutant of DC47
DC81	*aceF10 fadR adh-81*	Ethanol-utilizing mutant of DC48
DC82	*aceF10 fadR adh-82*	Ethanol-utilizing mutant of DC48
DC181	*aceF10 fadR adh-80* Δ(*tonB-trp*)	Spontaneous *tonB/trp* deletion of DC80
DC182	*aceF10 fadR adh-80* Δ(*tonB-trp*)	Spontaneous *tonB/trp* deletion of DC80
DC221	trp^+ *cysB* derivative of DC181	P1(DC172) × DC181
DC280	*aceF10 fadR trp adh-80*	P1(DC80) × DC244 for *adh*
DC281	*aceF10 fadR trp adh-81*	P1(DC81) × DC244 for *adh*
DC340	*recA srl*::Tn*10* derivative of DC280	P1(NK6659) × DC280
DC341	*recA srl*::Tn*10* derivative of DC281	P1(NK6659) × DC281

[a] CGSC indicates that the strain was received from the Coli Genetic Stock Center through the kindness of the curator, Barbara Bachmann.

For anaerobic growth, rich broth was supplemented with 1% glucose, 10 mM $KHCO_3$, 0.1 mM $FeSO_4$, 0.01 mM Na_2MoO_4, 0.01 mM H_2SeO_3, and 5 μg of thiamine per ml. Sodium nitrate (0.1%) and Tris (100 mM, pH 8.0) were added in some anaerobic experiments. Screw-capped serum bottles (200 ml) were filled completely with medium and incubated without shaking.

Genetic techniques. Mutagenesis was performed by adding ethyl methane sulfonate at a final concentration of 2% (vol/vol) or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at 100 μg/ml directly to late-exponential-phase cultures and incubating for 15 min at 37°C without shaking.

Initial mapping used the F'-mediated mobilization of the chromosome (9). F42 lac^+ and F15 $thyA^+$ were used. Transductions, using P1 *vir*, were performed as previously described (2). The *chlC* gene was scored by staining colonies for nitrate reductase activity (4).

Enzyme assays. Bacteria were grown in 200-ml batches to approximately 10^9/ml and harvested by

centrifugation. After washing with 50 mM potassium phosphate (pH 7.4), the cell pellet was suspended in 2.5 ml of this buffer and passed through a French pressure cell (Aminco) at 20,000 lb/in^2. The crude supernatant fraction was obtained by centrifugation of the homogenate for 30 min at 27,000 × *g* to remove unbroken cells and debris. A further centrifugation at 145,000 × *g* for 60 min gave the high-speed supernatant (soluble fraction). ADH was found almost entirely in the soluble fraction, whereas NR was present only in the crude supernatant.

ADH was assayed spectrophotometrically by following the production of $NADH^+$ at 340 nm (16). The cuvette contained NAD (75 nmol), ethanol (20 μl), enzyme preparation (5 to 50 μl), and 12 mM sodium pyrophosphate (pH 8.5) to give a final volume of 1.0 ml.

NR was assayed by coupling to the oxidation of methyl viologen (11). Dithiothreitol was omitted from the assay since it had no effect in crude cell extracts.

A unit of enzyme activity is defined as a nanomole of product formed per minute of incubation at 22°C.

RESULTS

Isolation of mutants. Late exponential cultures of the acetate auxotrophs DC47 and DC48 were mutagenized. Strain DC47 was grown in M9 medium with succinate plus acetate as carbon source and treated with ethyl methane sulfonate, whereas strain DC48 was grown in rich broth and treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine. After washing in M9, survivors were suspended at 10^7 viable cells per ml in 10-ml samples of medium containing succinate plus ethanol as the carbon source. Seven of 10 mutagenized cultures showed growth after 2 days of incubation at 37°C. Since ace^+ revertants would be selected by this procedure, a penicillin screening step in the absence of acetate (and ethanol) was included. After inoculation at 5×10^7 cells per ml into medium containing only succinate as the carbon source, cultures were treated with 4 mg of penicillin G per ml for 4 h. After centrifugation and washing to remove the penicillin, survivors were plated onto minimal agar containing succinate plus acetate and then replicated to media containing succinate alone, succinate plus acetate, or succinate plus ethanol. Of the seven suspect cultures, four contained only ace^+ revertants, two contained mutants capable of using ethanol as an acetate source, and one contained both types of strain.

Strain DC80 (derived from strain DC47) can use ethanol as an acetate source but not as a sole carbon source. Strains DC81 and DC82 were independently derived from strain DC48 and can use ethanol both to satisfy their acetate requirement and as a sole carbon source. Since growth on ethanol as the sole carbon source is feeble, most subsequent genetic manipulations employed ethanol only as an acetate source.

During the mutant isolation, fatty acyl derivatives were present, dissolved in the ethanol added to the media. The original object was to isolate mutants capable of using these fatty acyl derivatives as an acetate source. However, subsequent checking of strains DC80, -81, and -82 showed that these mutants were using the ethanol rather than the fatty acid derivatives as the acetate source.

Mapping of the *adh* locus. To map the *adh* mutation we constructed a series of recipient strains carrying the *aceF* and *fadR* mutations (see Materials and Methods). The presence of *aceF* is necessary to demonstrate the phenotype of the *adh* mutation in strain DC80. For strains DC81 and DC82, which can use ethanol as the sole carbon source, the presence of *aceF* is not strictly required, but it simplifies the scoring of recombinants since growth of strains DC81, DC82, and derivatives on ethanol as the sole carbon source is extremely slow. Furthermore, transfer of the mutations from strain DC81 or DC82 to certain genetic backgrounds gives mutants unable to use ethanol as a sole carbon source.

Preliminary experiments suggested that *fadR* was necessary to demonstrate the Adh phenotype. Several "revertants" of strain DC81 were found which no longer used ethanol as an acetate source, yet retained high levels of ADH. These strains were $fadR^+$ and could transfer out the *adh* mutation. However, later transduction experiments showed that the presence of *fadR* was not necessary for the expression of the Adh phenotype, at least in other genetic backgrounds.

F′-mediated chromosome mobilization (9) was used as an initial mapping step. Strain DC811, a spontaneous *thyA* mutant of strain DC81, was isolated by selection for trimethoprim resistance (18). DC813, a *lac* strain, was isolated by treating DC81 with ethyl methane sulfonate and plating on lactase-MacConkey agar (13). F15 thy^+ and F42 lac^+ were introduced into DC811 and DC813, respectively, and the resulting F′ derivatives were crossed with the multiply marked recipient strain DC23. Recombinants were selected at a variety of loci, and cotransfer of *adh* was found to be highest with the *trp* locus (69 and 74% using F15/DC811 and F42/DC813, respectively).

Cotransduction experiments were then performed using P1 grown on the *adh* mutants and recipient strains with markers in the region around *trp*. The mutations of strains DC80, DC81, and DC82 were all cotransduced with the *pyrF*, *cysB*, *trp*, and *hemA* loci, but not with the

purB locus (Table 2). These data indicate a location between *trp* and *hemA*. When samples of a given transduction mixture were plated on media containing succinate plus ethanol as carbon source, to select for *adh*, many fewer (10- to 100-fold) colonies were obtained than when markers such as *trp*$^+$ were selected. Addition of 0.05% casein hydrolysate improved the yield of *adh* transductants somewhat, but only for certain combinations of donor and recipient strains. Despite these technical difficulties, the reciprocal cotransduction frequencies observed for *adh* with *trp* and *cysB* are very similar (Table 2).

To locate *adh* more closely, spontaneous *tonB-trp* deletions were induced in strain DC80 by joint selection for resistance to ϕ80 and colicin V. The method used was essentially that of Gottesman and Beckwith (5), except that a mixed lysate containing colicins V and B and ϕ80 was prepared by UV induction of strain CSH48 (ϕ80 ColVB *trp*$^+$). Eight *trp* mutants were found out of 700 *tonB* isolates examined. All eight *trp-tonB* deletions retained the ability to use ethanol as an acetate source. This finding suggests that the *adh* locus is between *tonB* and *hemA*. Further localization of *adh* was attempted by means of three-point crosses using the *trp*, *adh*, and *chlC* loci (Table 3). The results indicate the gene order *trp adh chlC*. In addition to the crosses shown in Table 3, strains DC281 (*adh-81 trp*) and DC282 (*adh-82 trp*) were infected with P1 phage grown on strains DLM1 (*chlC*) or TS9A [*chlC*(Ts)]. However, no clear-cut four-crossover class was observed. Further investigation showed that many of the recombinants originally scored as *adh* were, in fact, *adh*$^+$. This mis-scoring is probably due to cross-feeding of *adh*$^+$ strains by *adh* colonies and does not occur when the *adh-80* allele is used, presumably because the *adh-80* mutation results in less overproduction of ADH than do the *adh-81* and *adh-82* alleles. The cotransduction frequencies of *chlC* with *adh* are 97, 96, and 95%, respectively, for the *adh-80*, *adh-81*, and *adh-82* alleles (Table 3), indicating that *adh* and *chlC* are extremely close.

The gene order in the *trp* to *purB* region of the chromosome including *adh* and neighboring markers is depicted in Fig. 1.

Enzymological characterization of the

TABLE 2. *Cotransduction of adh and nearby loci*

P1 donor	Recipient[a]	Marker selected	No. of colonies scored	% Cotransduction of donor marker
DC80 *adh*	DC25	*purB*$^+$	72	0
DC81 *adh*	DC25	*purB*$^+$	100	0
DC82 *adh*	DC25	*purB*$^+$	46	0
DC80 *adh*	DC31	*purB*$^+$	100	0
DC81 *adh*	DC31, DC39	*purB*$^+$	452	0
DC82 *adh*	DC31	*purB*$^+$	100	0
DC80 *adh*	DC185	*hemA*$^+$	218	25.2
DC81 *adh*	DC185	*hemA*$^+$	128	38.2
DC82 *adh*	DC185	*hemA*$^+$	86	30.2
DC80 *adh*	DC21	*trp*$^+$	177	21.5
DC81 *adh*	DC21	*trp*$^+$	200	39.5
DC82 *adh*	DC21	*trp*$^+$	28	35.7
DC80 *adh*	DC33, DC38	*trp*$^+$	188	49.5
DC81 *adh*	DC33, DC38	*trp*$^+$	198	48.5
DC82 *adh*	DC33, DC38	*trp*$^+$	138	32.6
DC80 *adh*	DC186	*cysB*$^+$	74	14.9
DC81 *adh*	DC186	*cysB*$^+$	100	33.0
DC82 *adh*	DC186	*cysB*$^+$	76	15.8
DC80 *adh*	DC68, DC69	*pyrF*$^+$	126	14.3
DC81 *adh*	DC68, DC69	*pyrF*$^+$	200	6.5
UB1005 *adh*$^+$	DC181	*trp*$^+$	41	53.7
UB1005 *adh*$^+$	DC182	*trp*$^+$	167	53.9
DC80 *trp*$^+$	DC33, DC244	*adh*	161	38.5
DC81 *trp*$^+$	DC33, DC244	*adh*	630	45.4
DC82 *trp*$^+$	DC33, DC244	*adh*	183	31.1
DC81 *cysB*$^+$	DC186	*adh*	100	44.0
DC82 *cysB*$^+$	DC186	*adh*	82	22.0
DC81 *hemA*$^+$	DC185	*adh*	29	41.4
DC82 *hemA*$^+$	DC185	*adh*	18	27.8

[a] In certain cases, two closely related recipients are grouped together. DC31/DC39, DC33/DC38, and DC68/DC69 differ only in that the lower-numbered strain carries the *aceF10* lesion, whereas the higher numbers carry the Δ*ace-15* lesion. DC244 is a spontaneous *gyrA* derivative of DC33.

TABLE 3. *Three-factor crosses*

P1 donor	Recipient	Selected marker	No. of mutants in recombinant class:			
			trp$^+$ *adh*$^+$	*trp*$^+$ *adh*	*trp adh*$^+$	*trp adh*
H680 *trp*	DC221 *adh-80 cysB*	*cysB*$^+$	1	519	9	71

P1 donor	Recipient	Selected marker	No. of mutants in recombinant class:			
			chl$^+$ *adh*$^+$	*chl*$^+$ *adh*	*chl adh*$^+$	*chl adh*
TS9A *chlC*	DC280 *adh-80 trp*	*trp*$^+$	19	46	32	3
DLM1 *chlC*	DC280 *adh-80 trp*	*trp*$^+$	45	124	76	21
DC80 *adh-80*	DC285 *chlC trp*	*trp*$^+$	6	94	180	30
DC81 *adh-81*	DC285 *chlC trp*	*trp*$^+$	5	128	127	26
DC82 *adh-82*	DC285 *chlC trp*	*trp*$^+$	4	48	83	20

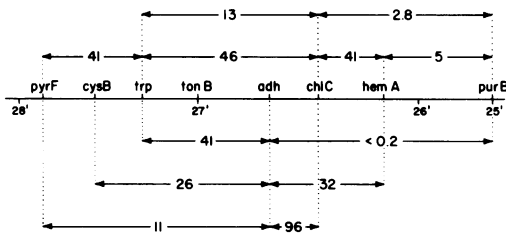

FIG. 1. *Genetic map of the pyrF to purB region. The linkages given are average cotransduction frequencies. Linkage data for adh are from Table 2. Data for other markers are from Guest (6) and this paper.*

***adh* mutation.** The first step in the biological oxidation of ethanol to acetate is its conversion to acetaldehyde by the enzyme ADH (1). We, therefore, assayed this enzyme in our wild-type and mutant strains. Table 4 gives the results of ADH assays performed on the high-speed supernatant fraction of cells grown on a variety of carbon sources. When grown on succinate plus acetate, the *adh* mutants overproduced ADH by 20-fold (strain DC80) or 60-fold (strain DC81 and DC82) relative to the wild-type strain. Growth with ethanol as an acetate source resulted in a doubling of the ADH activity of strains DC81 and DC82, although no effect was seen in strain DC80. However, when strain DC82 was grown with ethanol as the sole carbon source, the ADH activity was the same as for cultures grown on succinate plus acetate. Growth under conditions of catabolite repression (glucose plus acetate as carbon source) had little effect on the ADH level in strain DC80, but caused some repression in strains DC81 and DC82. Strains DC81 and DC82 can use ethanol as a sole carbon source, whereas strain DC80 cannot. The extent of the ability to use ethanol thus corresponds to the different levels of ADH found in these strains.

To find the lowest level of ADH sufficient to allow an *aceF* strain to use ethanol as an acetate source, revertants of strain DC80 were isolated. After treatment of strain DC80 with ethyl methane sulfonate, survivors were plated onto rich broth agar and then replicated to minimal plates containing succinate alone, succinate plus acetate, and succinate plus ethanol. Three revertants were found that retained the *aceF* mutation but were no longer able to use ethanol as an acetate source. The ADH activities of these relative to strain DC80 were strain DC805 (25%), strain DC806 (46%), and strain DC807 (60%) for cultures grown on succinate plus acetate. Apparently a twofold change in ADH production can determine whether or not a strain is able to use ethanol to fulfill its acetate requirement.

Further evidence that the increased production of ADH and the ability to use ethanol were due to the same genetic lesion was obtained by examining ADH levels in transductants. Transductants were obtained from infecting strains DC33 (*aceF trp*) or DC38 (*Δace-15 trp*) with phage P1 grown on the *adh* strains. Those transductants unable to use ethanol as an acetate source showed ADH levels of <1 U/mg (assays were performed on the crude supernatant fraction of cells grown in rich broth). Those transductants that were able to use ethanol and were derived from DC80 had ADH levels of 15 to 30 U/mg, whereas the transductants derived from DC81 or DC82 had levels of 70 to 200 U/mg and were able to use ethanol not only as an acetate source but also as the sole carbon source.

The ADH of strain DC81 resembles ADH activities from other organisms in respect to substrate specificity (1). Primary alcohols with 2 to 8 carbon atoms are good substrates; benzyl

TABLE 4. *ADH activities[a] in wild-type and mutant strains*

Strain	Aerobic				Anaerobic			
	Succinate + acetate	Glucose + acetate	Succinate + ethanol	Succinate + acetate + NO_3	RBG[b]	RBG + NO_3	RBG + Tris[c]	RBG + Tris + NO_3
DC47	1.7	2.3	NG[d]	<0.4	1.2	<0.4	4.2	0.95
DC80	32.7	27.9	31.3	25.7	2.0	<0.4	35.0	3.0
DC48	1.9	2.3	NG	<0.4	0.85	<0.4	3.3	3.9
DC81	119	55.3	194	32.5	7.3	<0.4	554	316
DC82	122	91.2	218[e]	37.1	ND[f]	ND	583	447

[a] ADH activities are given in units per milligram of protein and refer to the high-speed supernatant fraction.
[b] RBG, Rich broth plus glucose and other supplements for anaerobic growth specified in the text.
[c] Tris (pH 8.0) was included to give a final concentration of 100 mM.
[d] NG, No growth on this carbon source.
[e] DC82 grown on ethanol as the sole carbon source gave a value of 102.
[f] ND, Not done.

alcohol, isoamyl alcohol, and glycerol show poor activity; methanol, isopropanol, and *tert*-butanol do not react significantly (data not shown). The enzyme is stable in concentrated protein solutions, but loses activity in a few days at −20°C if diluted into phosphate buffer at low protein concentrations (≤0.5 mg/ml). The yeast enzyme shows similar instability at low protein concentrations (1).

Control of ADH in *E. coli*. *E. coli* ferments glucose to a variety of products under anaerobic conditions (17). Under mildly alkaline conditions (pH 8), at low phosphate concentration, and in the absence of nitrate, the major products are an approximately equimolar mixture of acetate and ethanol together with smaller amounts of succinate. When provided with nitrate, anaerobic *E. coli* can couple NAD(P)H oxidation to nitrate reduction (8) and does not reduce acetate to ethanol or fumarate to succinate. Fumarate reductase (which is responsible for the anerobic production of succinate) is repressed both by aerobic growth and, anaerobically, by the presence of nitrate (21). We, therefore, examined the response to ADH to anaerobic growth and to the presence of nitrate.

As shown in Table 4, ADH was strongly repressed by the presence of nitrate in both aerobic and anaerobic cultures except in the case of strain DC80 growing aerobically. However, ADH does not appear to be significantly induced by anaerobic conditions, and indeed, the overproduction of ADH by the *adh* mutants was reduced approximately threefold when these strains were grown anaerobically. However, when Tris buffer (100 mM, pH 8.0) was added to anaerobic cultures to prevent acidification, a large increase in ADH levels was observed. This correlates with the observation that fermentation under acidic conditions tends to produce lactic acid, whereas more alkaline conditions result in the accumulation of acetate plus ethanol (17).

NR. Since ADH is repressed by nitrate, and since *adh* maps very close to *chlC*, the structural gene for one of the NR subunits, we assayed NR activity in our strains. When grown aerobically with succinate plus acetate or succinate plus ethanol as the carbon source, all strains produced negligible amounts of NR (≤1 U/mg of protein in crude supernatant extracts). The activities of NR observed in anaerobically grown cells are given in Table 5. The *adh* mutants DC81 and DC82 produced approximately 25% of the wild-type level of NR when grown in the absence of nitrate, and approximately 50% when nitrate was present. Fullest induction of NR is expected in minimal medium with a nonfermentable carbon source such as glycerol. Under such conditions there is negligible difference between strain DC81 and its parent DC48. In contrast, strain DC80 (*adh*) appears to have slightly elevated levels of NR in rich media.

TABLE 5. *NR activities*[a]

Strain	RBG[b] + Tris	RBG + Tris + nitrate	Glycerol + Nitrate
DC47	6.1	350	371
DC80	9.6	540	296
DC48	5.0	392	625
DC81	0.9	200	502
DC82	1.6	176	ND[c]

[a] NR activities are given in units per milligram of protein and refer to the crude supernatant fraction of anaerobically grown cultures.

[b] RBG, See Table 4, footnote *b*. Values for cultures grown without Tris were not significantly different from those shown.

[c] ND, Not done.

Diploidy at the *adh* locus. In preliminary experiments F125, which covers the *trp-purB* region of the *E. coli* chromosome, was transferred into strains DC280 (*adh-80 trp*) and DC281 (*adh-81 trp*) using *trp* as selective marker. The F125$^+$ merodiploids appeared to have only half as much ADH as the parental strains. Since long F-prime factors such as F125 are frequently unstable (10), we repeated these experiments in the *recA* derivatives DC340 (DC280 *recA*) and DC341 (DC281 *recA*). In these *recA* strains, the level of ADH was the same for the F$^-$ and F125$^+$ derivatives. Thus the *adh* mutation appears to be dominant over the wild-type allele.

Propanediol dehydrogenase. Mutants of *E. coli* able to use 1,2-propanediol as a carbon source (*prd* mutants) produce a constitutive dehydrogenase able to act on 1,2-diols and ethanol (7). However, *prd* and *adh* map at quite different locations. Moreover, *aceF*$^+$ derivatives of *adh* mutants cannot use propanediol as carbon source. Thus, *adh* is distinct from the *prd* mutation.

DISCUSSION

A major problem of anaerobically growing *E. coli* is the disposal of excess reducing equivalents generated during the fermentation process. In the absence of nitrate, NAD(P)H is reoxidized by reducing pyruvate to lactate (acidic conditions), acetate to ethanol (alkaline conditions), or fumarate to succinate (at both acid and alkaline pH) (17). In the presence of nitrate, NAD(P)H oxidation is coupled via an electron transport chain to the reduction of nitrate (reviewed in 8). Anaerobic respiration using nitrate is more efficient than fermentation since it is no longer necessary to discard carbon in the form of reduced fermentation products, and, moreover, the electron transport chain that termi-

nates with NR is coupled to oxidative phosphorylation (8). We might expect that the various anaerobic redox systems would be regulated so as to optimize use of the available carbon source.

We have isolated mutants which map extremely close to an NR gene and in which ADH is overproduced by 20 to 60 fold. These mutants also show altered levels of NR. Strains DC81 and DC82 (ADH up 60-fold) show a fourfold decrease in NR. This deficiency is reduced by conditions which strongly induce NR. Furthermore, ADH is repressed by nitrate, both aerobically and anaerobically, both in the wild type and in strains DC81 and DC82. Mutant DC80 (ADH up 20-fold) differs from strains DC81 and DC82 in several respects. First, in strain DC80, the level of NR is increased by approximately 50%, and ADH is repressed by nitrate only under anaerobic conditions. Second, unlike strains DC81 and DC82, strain DC80 shows no increased level of ADH when grown in the presence of ethanol. Third, the level of ADH in strain DC80 is not decreased by the presence of glucose (Table 4).

Diploid analysis indicated that the *adh* mutations from both strains DC80 and DC81 were dominant, suggesting alterations in a transcriptional control site (such as an operator or promoter) rather than inactivation of a control protein. Although the pleiotropic effects of the *adh* mutations appear complex, one simple explanation is to propose the existence of an "anaerobic control protein," which has a central role in controlling anaerobic versus aerobic metabolism. Alteration of the transcriptional control of such a regulatory protein could cause pleiotropic changes in a variety of anaerobic systems. Further investigation along the lines of such a hypothesis would require the isolation of additional mutations affecting the same control system but in a different manner.

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