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Evolution of the *adhE* Gene Product of *Escherichia coli* from a Functional Reductase to a Dehydrogenase

GENETIC AND BIOCHEMICAL STUDIES OF THE MUTANT PROTEINS*

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Jorge Membrillo-Hernández‡§, Pedro Echave¶‖, Elisa Cabiscol¶, Jordi Tamarit¶, Joaquim Ros¶‡‡, and Edmund C. C. Lin‡**

From the ‡Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115 and ¶Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, 25198 Lleida, Spain

The multifunctional AdhE protein of *Escherichia coli* (encoded by the *adhE* gene) physiologically catalyzes the sequential reduction of acetyl-CoA to acetaldehyde and then to ethanol under fermentative conditions. The NH_2-terminal region of the AdhE protein is highly homologous to aldehyde:NAD^+ oxidoreductases, whereas the COOH-terminal region is homologous to a family of Fe^{2+}-dependent ethanol:NAD^+ oxidoreductases. This fusion protein also functions as a pyruvate formate lyase deactivase. *E. coli* cannot grow aerobically on ethanol as the sole carbon and energy source because of inadequate rate of *adhE* transcription and the vulnerability of the AdhE protein to metal-catalyzed oxidation. In this study, we characterized 16 independent two-step mutants with acquired and improved aerobic growth ability on ethanol. The AdhE proteins in these mutants catalyzed the sequential oxidation of ethanol to acetaldehyde and to acetyl-CoA. All first stage mutants grew on ethanol with a doubling time of about 240 min. Sequence analysis of a randomly chosen mutant revealed an Ala-267 → Thr substitution in the acetaldehyde:NAD^+ oxidoreductase domain of AdhE. All second stage mutants grew on ethanol with a doubling time of about 90 min, and all of them produced an $AdhE^{A267T/E568K}$. Purified $AdhE^{A267T}$ and $AdhE^{A267T/E568K}$ showed highly elevated acetaldehyde dehydrogenase activities. It therefore appears that when AdhE catalyzes the two sequential reactions in the counter-physiological direction, acetaldehyde dehydrogenation is the rate-limiting step. Both mutant proteins were more thermosensitive than the wild-type protein, but $AdhE^{A267T/E568K}$ was more thermal stable than $AdhE^{A267T}$. Since both mutant enzymes exhibited similar kinetic properties, the second mutation probably conferred an increased growth rate on ethanol by stabilizing $AdhE^{A267T}$.

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§ Recipient of The Bernard D. Davis Fellowship. Present address: Dept. de Biología Molecular, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, P. O. Box 70-228, 04510 Mexico City, México.

‖ Recipient of a Ph.D. fellowship from the Ministerio de Educación y Cultura (Spain).

** Recipient of a postdoctoral fellowship from the Generalitat de Catalunya (Spain).

‡‡ To whom correspondence should be addressed. Tel.: 34 973 702 407; Fax: 34 973 702 426.

When lacking molecular oxygen or other exogenous electron acceptors, *Escherichia coli* carries out mixed acid fermentation during anaerobic growth in order to achieve metabolic redox balance. The fermentation products include ethanol, formate, acetate, glycerol, D-lactate, succinate, CO_2, and H_2 (1, 2). As indicated by Reactions 1 and 2 below, ethanol arises from acetyl-CoA by two sequential NADH-dependent reductions catalyzed by the multifunctional ethanol oxidoreductase (the *adhE* gene product) comprising 891 amino acids (Refs. 3 and 4; see Fig. 1):

$$CH_3CO\text{-}SCoA + NADH + H^+ \overset{AdhE}{\rightleftharpoons} CH_3CHO + CoA + NAD^+$$

REACTION 1

$$CH_3CHO + NADH + H^+ \overset{AdhE}{\rightleftharpoons} CH_3CH_2OH + NAD^+$$

REACTION 2

AdhE appears to be the evolutionary product of a gene fusion. The NH_2-terminal region of this protein is highly homologous to the family of aldehyde:NAD^+ oxidoreductases, whereas the COOH-terminal region is homologous to the family of Fe^{2+}-dependent alcohol:NAD^+ oxidoreductases (3–5). Despite the fact that both AdhE-catalyzed reactions are reversible, *E. coli* fails to grow on ethanol as a sole carbon and energy source apparently for two main reasons. First, the *adhE* gene is insufficiently expressed under aerobic conditions (6–8). Second, the catalytic half-life of the AdhE protein is shortened during aerobic metabolism by a metal-catalyzed oxidation (MCO)[1] cycle. In this disabling process, the amino acid chains of AdhE are thought to be covalently attacked by the highly reactive hydroxyl radicals locally generated by the Fe^{2+} bound to the active site of the alcohol:NAD^+ oxidoreductase domain. In fact, AdhE has been identified as one of the major targets of protein oxidation in *E. coli* (9, 10).

The case of the *adhE* gene product and its role in general fermentation is analogous to that of the *fucO* gene product and its role in specific L-fucose fermentation (11). Unlike AdhE, FucO is not a fusion protein but a simple enzyme that belongs to the family of Fe^{2+}-dependent alcohol:NAD(P)$^+$ oxidoreductases and catalyzes physiologically the reduction of L-lactaldehyde to L-1,2-propanediol (11–13). Like AdhE, FucO also fails to serve as a dehydrogenase for aerobic growth, because the gene is inadequately expressed, and the enzyme is highly sus-

[1] The abbreviations used are: AdhE, ethanol oxidoreductase encoded by *adhE*; FucO, L-1,2-propanediol oxidoreductase encoded by *fucO*; MCO, metal catalyzed oxidation; MOPS, morpholinepropanesulfonic acid; kb, kilobase pair; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

TABLE I
Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant genotype	Source or Ref.
Strains		
CAG12169	*zci-506*::Tn*10*	M. Berlyn
DC272	*fadR mel tyrT adhC81*	8
ECL3999	MC4100 but *adhE*::*kan*	30
ECL4000	MC4100 Φ(*adhE-lacZ*)	30
ECL4060	ECL4000 but *adhC81*	ECL4000XP1(DC272)
ECL4063	ECL399 *attλ*::$adhE^{A267T}$	This study
ECL4064	ECL399 *attλ*::$adhE^{A267T}$	This study
ECL4065	ECL399 *attλ*::$adhE^{E568K}$	This study
ECL4066	ECL399 *attλ*::$adhE^{A267T/E568K}$	This study
JE21	ECL4000 but able to grow on ethanol	This study
JE29	ECL4000 but able to grow on ethanol	This study
JE46	ECL4000 transductant (back-crossed) of JE21	ECL4000XP1(JE21)
JE52	ECL4000 transductant (back-crossed) of JE29	ECL4000XP1(JE29)
MC4100	Δ(*argF-lac*)*U169 rpsL150 araD169 relA1 flb-5301 deoC1 ptsF25*	M. Casadaban
Plasmids		
PJMADH1	pBR322/$adhE^{+}$ from MC4100	30
PJMADH4	pBR322/$adhE^{A267T}$	This study
PJMADH5	pBR322/$adhE^{E568K}$	This study
PJMADH6	pBR322/$adhE^{A267T/E568K}$	This study
pRS415	Ap^{r} operon fusion vector	25
Bacteriophages		
λRS45	Specialized phage for recombination with pRS vectors	25
λADHop656	(*adhE-lacZ*) Comprising up to 656 base pairs of the promoter region of *adhE* from MC4100	26

ceptible to MCO during aerobic metabolism. We have previously characterized *E. coli* mutants that acquired aerobic growth ability on L-1,2-propanediol by recruiting FucO to serve as a dehydrogenase (Ref. 14 and references therein). Two kinds of mutations contributed to such an ability. First, an IS*5* insertion occurred in the *fucO* promoter that resulted in high constitutive expression of the *fucAO* operon (15). Second, a missense mutation occurred that conferred resistance of FucO to MCO (9, 14, 16).

A mutant that grew on ethanol as sole carbon and energy source was previously reported (6). However, the nature of the mutation(s) responsible for the ethanol^{+} phenotype was not definitively determined. Here we report the isolation of 16 series of independent ethanol^{+} mutants and the characterization of the genetic changes at the molecular level. Our results showed that the evolution of AdhE as a dehydrogenase followed a strategy that differs from that of FucO.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions, and Preparation of Cell Extracts—The relevant characteristics and sources of bacterial strains, plasmids, and phages used in this study are given in Table I. Luria Bertani (LB) medium containing 0.1 M MOPS and 0.2% glucose was adjusted to pH 7.4 (LB-glucose medium). Minimal medium was prepared as described previously (10) and was supplemented with 0.2% glucose or 2% ethanol as carbon and energy source. Solid media contained 1.5% Bacto-agar (Difco). Culture absorbance (A_{600}) was determined in a DU640 Beckman spectrophotometer. Aerobic 10-ml cultures were grown at 37 °C in 250-ml Erlenmeyer flask shaken at 250 rpm. Anaerobic cultures were grown at 37 °C in 100-ml flasks filled to the brim. For anaerobic growth on solid media, a Gas-Pak system was used. When appropriate, antibiotics (Sigma) were added at the following concentrations: 200 μg ampicillin/ml and 10 μg tetracycline/ml.

For enzyme assays of cell extracts, cultures during mid-exponential phase of growth were harvested and prepared as described previously (17). The cell pellets were suspended in four times their wet weight in 50 mM Tris-HCl (pH 8.5), 50 mM KCl, 5 mM dithiothreitol, 2 mM NAD, and protease inhibitors 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, and 1 μM pepstatin A. The suspended cells were disrupted by sonication. Samples were centrifuged at 12,000 × *g* for 30 min at 4 °C, and the supernatant was used for assays.

Enzyme Assays—To determine the level of AdhE activities, the cells were grown on glucose or ethanol minimal mineral medium. Ethanol dehydrogenase activity was assayed in 1.5 M ethanol, 2.5 mM NAD, and 300 mM potassium carbonate at pH 10.0. Acetaldehyde dehydrogenase activity was assayed in 100 mM acetaldehyde (10 mM for purified enzymes), 2.5 mM CoASH, 2.5 mM NAD, and 300 mM potassium carbonate at pH 10.0 (18). The rates of both dehydrogenase reactions were monitored by the formation of NADH at 340 nm (19). Acetyl-CoA reductase activity was assayed in 1 mM acetyl-CoA, 0.25 mM NADH, and 50 mM Tris-HCl (pH 7.5). Acetaldehyde reductase activity was assayed in 100 mM acetaldehyde (10 mM for purified enzymes), 0.25 mM NADH, and 50 mM Tris-HCl (pH 7.5) (18). The rate of both reductase reactions was monitored by the disappearance of NADH at 340 nm (19). In all of the assays 1 activity unit corresponds to 1 μmol of substrate converted per min. β-Galactosidase activity was assayed as described previously by Miller (20).

Purification of AdhE Wild-type and Mutant Proteins—6 liters of anaerobic cultures were grown overnight at 37 °C in minimal medium containing 0.2% glucose. Cultures were centrifuged and disrupted by two 1-min cycles of sonication with 1-min intervals of resting on ice. Cell-free extracts were obtained by centrifugation at 15,000 × *g* for 30 min at 4 °C. Ammonium sulfate was slowly added to the extracts being stirred and chilled on ice until 20.6% saturation was reached. The mixture was left chilled for 30 min and then centrifuged for 15 min at 4 °C at 15,000 × *g*. The supernatant fraction was recovered, and ammonium sulfate was added until 30% saturation while being stirred and chilled on ice. After 30 min of equilibration, the mixture was again centrifuged, as described above. This time the pellet was recovered and resuspended in 2.5 ml of 50 mM Tris-HCl (pH 8.5) (approximately 30–35 mg of protein/ml). The sample was applied to an Ultrogel AcA44 gel filtration column (IBF Biotechnics, Paris, France) pre-equilibrated with 100 mM KCl, 50 mM MOPS-KOH at pH 7.65. Elution with the same buffer was carried out at a flow rate of 1.4 ml/min. Fractions showing significant AdhE activity were pooled and diluted (1:1 v/v) with 50 mM Tris-HCl (pH 8.5). The sample was then loaded into a DEAE-15HR column (Waters Associates, Milford, MA) equilibrated with 50 mM Tris-HCl (pH 8.5) and 50 mM NaCl. After a washing step of 30 min with the same buffer, elution was carried out by a linear gradient of 50–200 mM NaCl at a flow rate of 5 ml/min for 20 min. Peak AdhE elution occurred at approximately 15 min. Purity of the samples was examined by SDS-polyacrylamide gel electrophoresis (21). Protein concentration was determined by the Bradford method (22), using bovine serum albumin as standard.

Thermal Stability Assays of AdhE—Purified wild-type and mutant AdhE proteins (0.14 mg/ml) were incubated at 37 °C. Samples were withdrawn at different time intervals for assay of ethanol dehydrogenase activity.

Oxidative Inactivation of AdhE—Purified wild-type and mutant AdhE proteins (0.14 mg/ml) were incubated at 15 °C either in the presence of 1 mM NADH or 2 mM ascorbate plus 50 μM $FeCl_3$ (23).

TABLE II
β-Galactosidase and ethanol dehydrogenase activities of cell extracts from strains ECL400, JE46, and JE52 under different growth conditions

Strain	Ethanol dehydrogenase activity[a]			β-Galactosidase activity[b]		
	Glucose + O_2	Glucose − O_2	Ethanol + O_2	Glucose + O_2	Glucose − O_2	Ethanol + O_2
ECL4000	0.08 ± 0.04	0.95 ± 0.04		970 ± 50	9700 ± 240	
JE46	0.43 ± 0.10	1.61 ± 0.20	0.78 ± 0.10	580 ± 30	7000 ± 160	540 ± 40
JE52	0.61 ± 0.10	1.98 ± 0.30	0.89 ± 0.10	520 ± 40	5300 ± 130	500 ± 60

[a] AdhE-specific activity is expressed as units/mg total protein.
[b] LacZ activity is expressed in Miller units (20).

Samples were withdrawn at different time intervals for assay of ethanol dehydrogenase activity.

Genetic Procedures for Analyzing adhE Mutant Alleles—Genetic crosses were performed by P1*vir*-mediated transduction (20). Standard methods were used for restriction endonuclease digestion and ligation of DNA (24, 25). Plasmid DNA was isolated by using the QIAprep system, and the DNA fragments were isolated from agarose gels with the QIA quick kit (Qiagen). Bacteria were transformed with plasmid DNA electroporation (24) with an *E. coli* Pulser (Bio-Rad). Polymerase chain reaction amplifications were carried out in a Minicycler (MJ Research), using *Pfu* DNA polymerase from Stratagene (La Jolla, CA). Oligonucleotides were custom-synthesized (DNA Integrated Technologies). Sequence determination of *adhE* alleles was carried out by amplifying four different fragments (A, B, C, and D) of the *adhE* gene using the following primers: A5 (5′-ATCACAGTGAGTGTGAGCGCGAGTAAGC-3′), A3 (5′-GCCAACTGCACGTTTGATATCAGC-3′), B5 (5′-GGTTTAAGCCGCATACAGCTCCGG-3′), B3 (5′-GCCATCAGTAATCACTTCATCC-3′), C5 (5′-CTCGCACCTTCCCTGACTCTGGG-3′), C3 (5′-CGTCCAAGACCACCGAAAGCACCACAGGG-3′), D5 (5′-CCTGGTTATGGACATGCCGAAG-3′), and D3 (5′-GAAGGGGCCGTTTATGTTG-3′). Each polymerase chain reaction fragment was subjected to automated DNA sequencing at the CORE facility at Harvard Medical School.

Site-directed Mutagenesis—Construction of plasmid pJMADH1 was described previously (26). Site-directed mutagenesis in the *adhE*-coding region was performed with the QuickChange kit (Stratagene). Primers T5 (5′-GTGAACGTTTT**A**CAACCCACGGCGGC-3′) and T3 (5′-GCCGCCGTGGGTTGT**T**AAAACGTTCAC-3′) were used to introduce the Ala-267 → Thr mutation in pJMADH4. Primers K5 (5′-GGAAACTCACTCGAA**A**AGCTGGCGCTGCGC-3′) and K3 (5′-GCGCAGCGCCAGCT**T**TTCGAGTGGAGTTTCC-3′) were used to introduce the Glu-568 → Lys mutation in pJMADH5. Plasmid pJMADH6, containing both mutations, was constructed by using plasmid pJMADH4 as a template with primers K5 and K3. Confirmation of the sequences of all the inserts was performed by automated DNA sequencing (CORE Facility at Harvard Medical School).

Insertion of Wild-type and Mutant adhE Alleles into Host Chromosomes via λ Vectors—The plasmids pJMADH1, pJMADH4, pJMADH5, and pJMADH6 were digested with restriction enzymes *Bam*HI and *Eco*RI to yield 3.8-kb fragments that contain a full-length *adhE* operon. These fragments were then ligated using T4 DNA ligase (Promega, Madison, WI) with wild-type λ DNA cut with the same enzymes. The ligation mixtures were assembled into complete phage particles by using the Gigapack Gold Lambda packaging extract (Stratagene, La Jolla. CA). Phage particles were used to transduce strain ECL3999 (*adhE*::kan). Single copy insertions of the *adhE* operon were confirmed by Southern blots probed with *adhE* fragments at both ends of the *adhE* sequence.

RESULTS

Selection of Mutants with Acquired Aerobic Growth Ability on Ethanol as a Sole Carbon and Energy Source—Since direct selection on minimal ethanol medium failed to yield the desired mutants, we used a two-step approach. About 100 cells of the merodiploid strain ECL4000 (*adhE*$^+$ Φ[*adhE-lacZ*]) were spread on each of 100 MacConkey base agar (Difco) plates containing 2% ethanol. The plates were then sealed with parafilm to retard evaporation and then incubated for 24 h at 37 °C. Up to this time, all the colonies were semi-transparent and colorless. After 6 days of incubation under sealed conditions, red papillae appeared on many colonies. On 5 control plates without ethanol, no red papillae appeared. Among about 10^4 colonies screened on the MacConkey-ethanol agar, 1425 colonies presented red papillae. At least one papilla from each of these 1425 colonies was streaked on the same kind of agar for purification. A single red colony from each streak was then tested for growth ability on agar containing mineral medium and 2% ethanol. For reasons undetermined, only 31 red clones were found to have an ethanol$^+$ phenotype. Sixteen independent ethanol$^+$ mutants were adopted for further study. The doubling time of all these mutants in liquid mineral medium containing 2% ethanol was about 240 min at 37 °C. A phage P1 lysate was then prepared from each of the mutants to transduce strain ECL4000 and selected for aerobic growth on ethanol. One ethanol$^+$ transductant (first stage mutants) from each transduction experiment was subjected to an additional 100 generations of selection in the same ethanol liquid medium. A sample of the cells from each line of selection was then plated again on ethanol agar, and one colony that clearly exhibited an increased growth rate was isolated. A phage P1 lysate was then prepared from each of the 16 clones with improved growth rate on ethanol to transduce strain ECL4000, and the transductants were selected for aerobic growth on ethanol. All backcrosses gave rise to the large colony size phenotype. When each of these back-crossed strains (second stage mutants) were grown at 37 °C in liquid mineral medium containing 2% ethanol, all showed a doubling time of about 90 min. These results indicate that the first and second mutations are transductionally linked. A first stage mutant, JE46, and a second stage mutant, JE52, were picked as representatives for both genetic and biochemical characterizations (Table II).

Indication of cis Mutations by Enzymatic Analysis of Merodiploid Evolvants—For a preliminary *cis/trans* test for the two mutations, we took advantage of the *adhE* Φ(*adhE-lacZ*) merodiploid background. A *cis* mutation in *adhE* should primarily increase the ethanol dehydrogenase activity level, whereas a *trans*-positive regulatory mutation should elevate the expression of both *adhE* and Φ(*adhE-lacZ*).

When wild-type and mutant cells were grown aerobically on glucose, ethanol dehydrogenase activities were found to be 5.3-fold elevated in strain JE46 and 7.6-fold elevated in strain JE52 when compared with the wild-type level. The levels of the dehydrogenase activity were even more elevated when the mutant cells were grown aerobically on ethanol (possibly because of substrate stabilization). Curiously, the β-galactosidase activity levels were found to be 40% lower in strain JE46 and 46% lower in strain JE52 when compared with the wild-type level (Table II). In any event, the increase in ethanol dehydrogenase level without concomitant increase in β-galactosidase activity level would suggest that both mutations acted in *cis*. According to Leonardo and co-workers (8), the change of β-galactosidase activity levels in a direction opposite to that of ethanol dehydrogenase is best explained as follows. A *cis* mutation was responsible for elevating the dehydrogenase activity levels in the mutants JE46 and JE52. The increase in this activity (consumption of NADH coupled with the reduction of acetyl-CoA) during aerobic growth on glucose would raise the cellular redox potential. Such a state would in turn cause a decrease in the synthesis of β-galactosidase under the direction of the *adhE* promoter. The *adhE* promoters of *Aerobacter aerogenes*

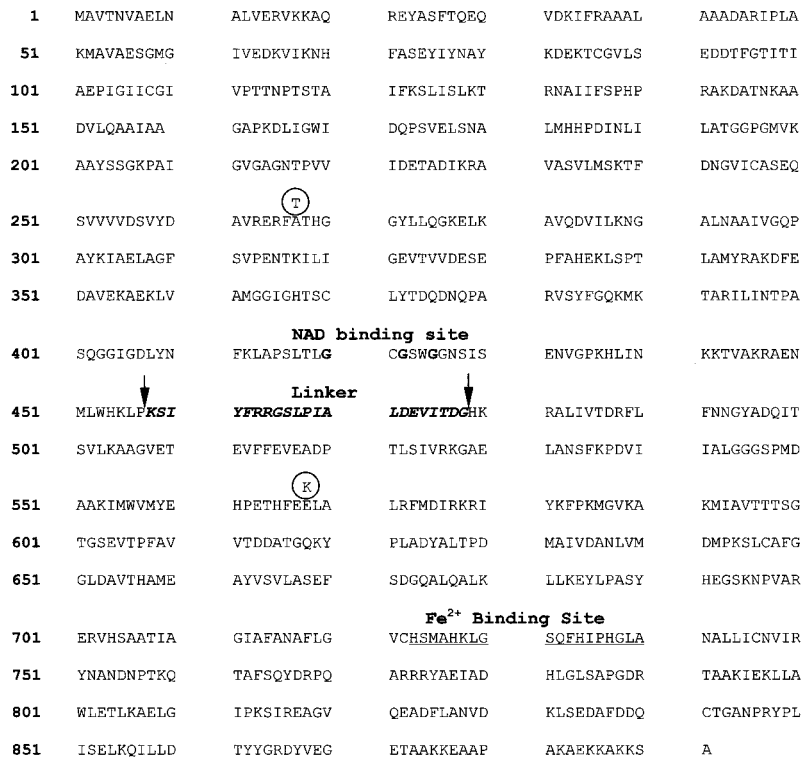

FIG. 1. **AdhE amino acid sequence and putative binding sites.** The acetaldehyde dehydrogenase and ethanol dehydrogenase domains are connected by a proposed linker (*bold italicized letters*) indicated by the *arrow* (33). The NAD-binding site is located on the basis of the GXGXXG motif. The iron-binding site (9) is shown with its conserved residues *underlined*. The substituted amino acid residues (*T* and *K*) in the mutant proteins are *encircled*.

(now classified as *Klebsiella pneumoniae*) and *E. coli* are thought to be activated by low cellular redox states, as reflected by high NADH/NAD ratios (7, 28, 29).

Further Evidence in Support of Mutations cis to adhE by Transduction Analysis—To confirm that the mutations in JE46 and JE52 were *cis* to the *adhE* locus situated at min 27.8 (31), we prepared a $P1_{vir}$ lysate from each of the mutants to transduce strain CAG12169 that bears *zci-506*::Tn*10* insertion at min 28 (31). If the mutation(s) responsible for the increase of *adhE* expression is indeed *cis*, then all of the transductants selected for the ability to grow on ethanol should lose the closely linked transposon Tn*10* that confers tetracycline resistance. When 2000 ethanol$^+$ transductants, obtained from each of the two $P1_{vir}$ lysates, were analyzed for the drug resistance, all were found to be Tet^s, affirming that the mutation(s) was in *cis*. There are several ways by which a mutation *cis* to *adhE* can increase the ethanol dehydrogenase activity level. For example, a regulatory mutation could increase the rate of transcription or translation of the gene, or a structural gene mutation may occur that enhances the catalytic activity or the half-life of the protein.

Sequence Determination of Mutant adhE Alleles—To locate the mutations, we sequenced each entire mutant gene from 1 kb upstream of the ATG codon to the end of the open reading frame (see "Experimental Procedures"). As expected, the promoter region of strains JE46 and JE52 was identical to that of the wild-type sequence previously reported (32). On the other hand, the coding region of the *adhE* gene in strain JE46 showed an A to G transition at position 799 from the A of the initiation codon ATG. This change converts amino acid residue 267 from Ala to Thr and is located inside the acetaldehyde dehydrogenase domain (Fig. 1). The coding region of the *adhE* gene in strain JE52 showed two base changes as follows: the same substitution found in strain JE46, plus an A to G transition at position 1702 that converts the amino acid residue 568 from Glu to Lys, which is located inside the ethanol dehydrogenase domain. Strikingly, when the 15 other independently isolated second stage mutants were also subjected to DNA sequence analysis, the same pair of mutations present in strain JE52 was found. It might be noted that the presence of the mutations considerably downstream of the sequence specifying the RBS site also makes translational control an unlikely mechanism for the increased ethanol dehydrogenase activity level in the mutant strains.

Reconstitution of adhE^{A267T} and adhE$^{A267T/E568K}$ Mutant Alleles—To demonstrate that the mutations identified in strains JE46 (*adhE*^{A267T}) and JE52 (*adhE*$^{A267T/E568K}$) are sufficient to account for their phenotypes, we prepared λ phage bearing wild-type or each of the two mutant alleles (see "Experimental Procedures") to lysogenize strain ECL3999 (*adhE*::kan). The control lysogen ECL4063 (*adhE*::kan λ*att*::*adhE*$^+$) showed aerobic and anaerobic levels of AdhE activity indistinguishable from those of the wild-type strains MC4100 and ECL4000. When strain ECL4064 (*adhE*::kan λ*att*::*adhE*^{A267T}) was tested, the activity levels were similar to those of strain JE46, and the cells grew on ethanol as a sole carbon and energy source at a rate similar to that of cells of JE46. Similarly, strain ECL4066 (*adhE*$^{A267T/E568K}$) showed the same phenotypes as strain JE52 (data not shown).

Identity of the Double Mutations in Strain JE52 and the Previously Isolated Strain DC272—Starting from an acetate auxotroph (*aceF10*, defective in dihydrolipoyltransacetylase component E2p), DC48, Clark and Cronan (6) isolated a nitrosoguanidine-induced mutant, DC81, that acquired the ability to use ethanol as a substitute of acetate. Strain DC81 also grew on ethanol as sole carbon and energy source. When grown aerobically on glucose and acetate, DC81 exhibited an ethanol dehydrogenase activity level more than 20-fold higher than that of DC48. It was suggested on the basis of genetic analysis that the mutation in DC81 altered a transcriptional site of the *adh* gene at min 27 (6), later referred to as the promoter constitutive mutation *adhC* (27). Strain DC272, re-examined in this study, was an *ace*$^+$ transductant of strain DC81 bearing that *adhC* mutation (28). Since all of the 16-second stage mutants possessed wild-type promoters and strains JE52 and DC272 grew at the same rate on ethanol at 37 °C (about 90-min

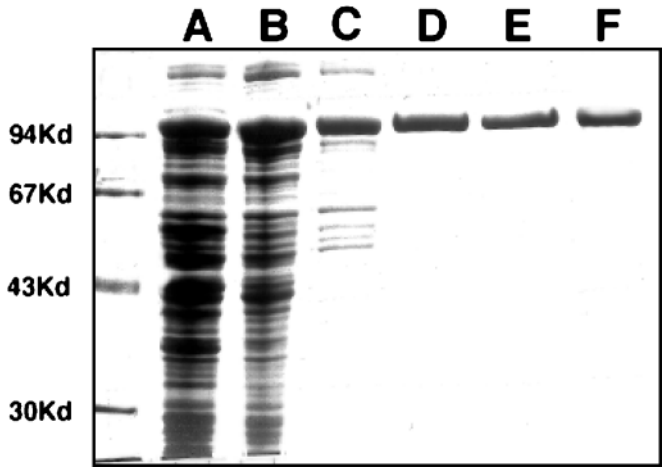

FIG. 2. **SDS-polyacrylamide gel electrophoresis analysis of the AdhE proteins.** Samples at various stages of purification were electrophoresed in 9% acrylamide. *A,* cell extract from strain MC41000; *B,* the preparation after the ammonium sulfate step; *C,* the preparation after the Ultrogel filtration step; *D,* the final preparation of AdhE after the liquid chromatography step; *E,* the final preparation of AdhE^{A267T} following the same procedure described for AdhE; *F,* the final preparation of AdhE$^{A267T/E568K}$ following the same procedure described for AdhE.

doubling time), we wondered what kind of mutation(s) took place in strain DC272 bearing the *adhC* locus (6, 28). The *adhC* allele comprising a 1-kb stretch upstream of the initiation codon and the entire open reading frame of *adhE* was therefore sequenced. To our surprise, the *adhE* allele of strain DC272 sustained exactly the same 2 base pair changes that occurred in JE52. No base change in the promoter region was found. To confirm that these two mutations were solely responsible for the ethanol$^+$ phenotype of strain DC272, we transduced the *adhC* allele into strain ECL4000 (the isogenic parent of JE52) to yield strain ECL4060. When strains JE52 and ECL4060 were grown in parallel aerobically on ethanol or anaerobically on glucose, the ethanol dehydrogenase activity levels were indistinguishable in the paired extracts (data not shown). We therefore conclude that the locus *adhC* is no other than *adhE*$^{A267T/E568K}$ allele (31).

Purification, Thermal Stability, and MCO Susceptibility of the AdhE, AdhE^{A267T}, and AdhE$^{A267T/E568K}$ Proteins—The three AdhE proteins from wild-type and mutant cell extracts were purified to electrophoretic homogeneity as shown in Fig. 2. The purified proteins were then compared for their thermal stability at 37 °C and pH 8.5 (Fig. 3). Not surprisingly, the wild-type AdhE protein was the most stable ($t_{½}$ = 35 min). Interestingly enough, AdhE^{A267T} was less stable ($t_{½}$ = 16 min) than AdhE$^{A267T/E568K}$ ($t_{½}$ = 32 min).

In the case of genetic mobilization of FucO, amino acid substitutions that conferred MCO resistance improved the aerobic growth ability on propanediol (14). To test the sensitivity of AdhE proteins to MCO damage, the oxidative inactivations of the three proteins were compared under two different conditions that promote the Fenton reaction. In the first experiment, the proteins were incubated in a solution containing Fe^{3+} as the metal ion and ascorbate as the reducing agent (Fig. 4*A*). In the second experiment, the inactivation was catalyzed by the enzyme bound Fe^{2+} as the metal ion and added NADH as the reducing agent (Fig. 4*B*). Under both conditions, the mutant proteins were more sensitive than the wild-type protein. Significantly, AdhE$^{A267T/E568K}$ was less sensitive than AdhE^{A267T}. The fact that the mutant protein with double amino acid substitutions was both more thermal stable and MCO-resistant than the mutant protein with a single mutation would suggest that the second E568K substitution stabilized the architectural integrity of the enzyme rather than improved the catalytic activity. These results are in contrast to those obtained with the FucO mutant proteins. In that case, FucO^{I7L} was more thermal stable but less MCO-resistant than FucO$^{I7L/L8V}$, thus indicating that the increased fitness of FucO for aerobic function was entirely dependent on MCO resistance.

Kinetic Analysis of Purified AdhE, AdhE^{A267T}, and AdhE$^{A267T/E568K}$ Proteins—Since the mutants were selected for more rapid utilization of ethanol, we compared the purified AdhE proteins for the two substrate-oxidizing reaction rates that the proteins catalyze the specific ethanol and acetaldehyde dehydrogenase activities. The ethanol dehydrogenase specific activities of purified AdhE^{A267T} or AdhE$^{A267T/E568K}$ were similar to each other but only 1.3-fold higher than that of AdhE (Table III). It might be recalled that the ethanol dehydrogenase specific activities of extracts from AdhE^{A267T} or AdhE$^{A267T/E568K}$ cells grown aerobically on glucose were 5.3–7.6-fold, respectively, higher than that of AdhE cell extracts (Table II). This discrepancy may in part be explained by significant inactivation of the mutant enzymes during the course of purification, as suggested by the stability data shown in Fig. 3. What is critical to note, however, is that the acetaldehyde dehydrogenase specific activities of purified AdhE^{A267T} and AdhE$^{A267T/E568K}$ were 5–6-fold higher than that of AdhE (Table III). To confirm the occurrence of enzyme inactivation during purification, we then examined acetaldehyde dehydrogenase specific activities of AdhE^{A267T} and AdhE$^{A267T/E568K}$ in extracts of cells grown aerobically on glucose. The specific activities were about 16–18-fold higher than that of AdhE (data not shown), supporting the notion that partial inactivation of the mutant enzymes occurred during the course of purification. Perhaps it is no coincidence that both mutant proteins contained an amino acid substitution in the acetaldehyde domain (Fig. 1). When the K_m values for ethanol and acetaldehyde were examined in the same pair of reactions, the values for the mutant proteins were indistinguishable from each other but were significantly lower than those for the wild-type protein (Table III).

DISCUSSION

The emergence of the AdhE fusion protein was probably a turning point in the evolution of the fermentative network of an ancestor of *E. coli* (33). From the perspective of catalysis, the fusion of an acetaldehyde oxidoreductase and an ethanol oxidoreductase probably accelerated the successive reduction of acetyl-CoA to ethanol by bringing the two active sites in close proximity. As a corollary, the steady state level of acetaldehyde, a toxic intermediate, could probably be lowered. It should be mentioned, however, that such a condition might also be achieved by the complexing of the two separate oxidoreductases, as in *Clostrium kluyveri* (34, 35).

Members of the family of aldehyde oxidoreductases have their NAD-binding sites near the COOH-terminal end, whereas members of the family of Fe^{2+}-dependent alcohol oxidoreductase have their NAD-binding sites near the NH_2-terminal end. Interestingly, a sequence analysis of AdhE of *E. coli* revealed only a single NAD-binding motif on the NH_2-terminal side of the linker (Fig. 1). If indeed there is only a single NAD-binding site, it is possible that evolution of the fusion not only brought the two catalytic sites close together but also made it possible to dispense with the coenzyme-binding site of the parent alcohol oxidoreductase. The sharing of the remaining NAD-binding site could in principle greatly facilitate the sequential catalysis. An added advantage of fusing the two proteins might provide the more elaborate structure of the protein with the potential to acquire other functions, such as the deactivation of pyruvate formate lyase (4). There may well

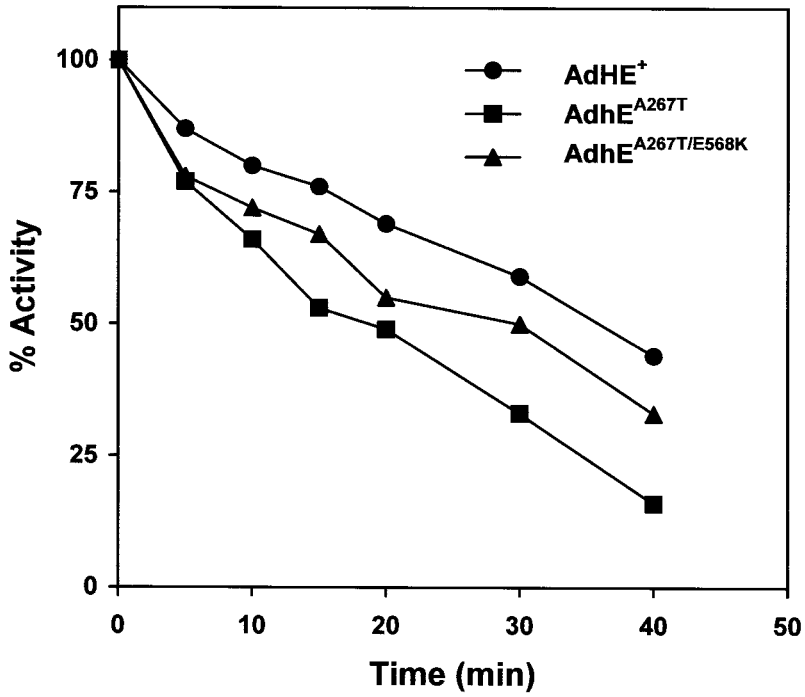

FIG. 3. **Thermal inactivation of different AdhE proteins.** The AdhE^{+} (●), AdhE^{A267T} (■), and AdhE$^{A267T/E568K}$ (▲) proteins were incubated in 50 mM Tris chloride, pH 8.5, and 160 mM NaCl at 37 °C. Samples for ethanol dehydrogenase activity measurements were taken at several time intervals. The results shown are the average of four independent experiments, and every point was done in triplicate; the variation was less than 15%.

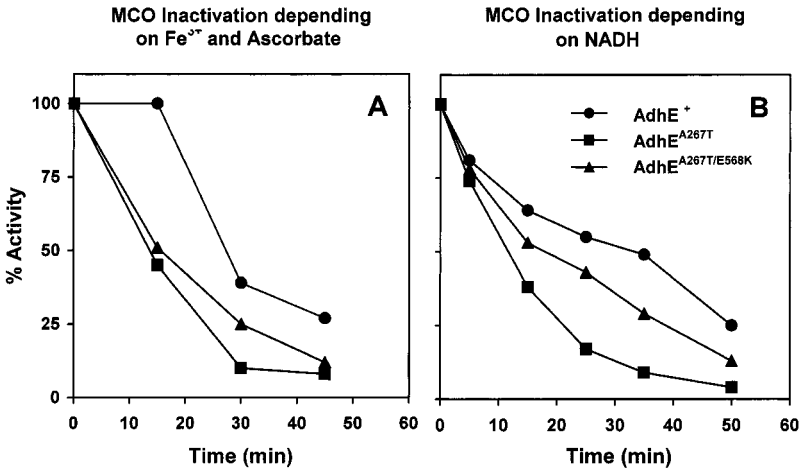

FIG. 4. **MCO inactivation of different AdhE proteins.** The AdhE^{+} (●), AdhE^{A267T} (■), and AdhE$^{A267T/E568K}$ (▲) were incubated in 50 mM Tris chloride (pH 8.5) and 160 mM NaCl in the presence of 2 mM ascorbate and 50 μM $FeCl_3$ (*A*) or 1 mM NADH (*B*) at 15 °C. Samples for ethanol dehydrogenase activity measurements were taken at several time intervals. The results shown are the average of three independent experiments, and every point was done in duplicate; the variation was less than 20%.

TABLE III
Specific enzymatic activities of purified AdhE proteins (units/mg AdhE) and K_m values for ethanol and acetaldehyde

The data shown are the average of three independent experiments with an S.D. of less than 20%.

Protein	Ethanol dehydrogenase	K_m ethanol	Acetaldehyde dehydrogenase	K_m acetaldehyde
		mM		*mM*
AdhE	10	240	4.0	5.4
AdhE^{A267T}	13	190	23	4.0
AdhE$^{A267T/E568K}$	13	190	20	4.0

be other accrued functions yet to be discovered. For instance, we do not yet know the biological significance of spirosomes consisting of AdhE molecules (4, 32).

The fact that all 17 independent ethanol^{+} mutants characterized in this study exhibited altered AdhE structures would indicate that promoter-up mutations were either extremely rare or deleterious. Indeed, when we plated MC4100 transformant cells bearing a multicopy plasmid (pBR322 derivative) containing the *adhE* gene under the control of an IPTG-inducible promoter on ethanol-IPTG agar, no growth was observed. Worse yet, when the transformant cells were grown aerobically on glucose in a mineral medium, the addition of IPTG was bactericidal. Thus, an excessive concentration of the AdhE protein appears to be toxic. Even a more moderate increase in the level of the protein seems to be detrimental, since transformant cells bearing the same multicopy plasmid containing the *adhE* gene under the control of its own promoter failed to grow aerobically on ethanol. Moreover, such cells were growth-impaired on glucose as the sole source of carbon and energy.[2] It is not clear whether the toxicity is related to the AdhE structure or its catalytic activity. Whatever the reason, the deleterious effect of excessive levels of AdhE protein under aerobic conditions may explain why up-regulated promoter mutations were not selected.

The striking increase in the specific acetaldehyde dehydrogenase activity relative to the specific ethanol dehydrogenase activity of AdhE^{A267T} would suggest that the second reaction was rate-limiting when AdhE was selected to catalyze the two sequential reactions in the direction opposite to the physiological one. As a consequence of the mutation, however, the protein appears to be destabilized. Since AdhE$^{A267T/E568K}$ exhib-

[2] J. Membrillo-Hernández, unpublished observations.

ited kinetic properties indistinguishable from those of $AdhE^{A267T}$ but showed increased stability *in vitro*, it would appear that the Glu-568 → Lys substitution raised the steady state level of cellular acetaldehyde dehydrogenase activity by partially stabilizing $AdhE^{A267T}$ (however, we cannot rule out the possibility that the Glu-568 → Lys mutation alone confers the ability to grow on ethanol).

An alternative strategy to raise the steady level of cellular acetaldehyde dehydrogenase activity would be to confer resistance of the AdhE protein to MCO damage. However, we failed to isolate such a mutant. Perhaps the Ala-267 → Thr and Glu-568 → Lys mutations conferred such large increases in the efficacy of the novel function of AdhE that the MCO-resistant mutations conferring only modest improvements failed to be selected. Finally, it should be mentioned that Ala-267 in the acetaldehyde oxidoreductase domain and Glu-568 in the ethanol oxidoreductase domain are not invariable amino acids within their respective conserved regions (data not shown). An observation that remains to be explored is the basis for the relatively small difference in AdhE activity level of mutant and wild-type cells grown anaerobically on glucose (Table II). An even more intriguing mystery is our failure to select for $AdhE^{A267T}$ directly on minimal ethanol agar.[2]

Mobilization of the *adhE*-encoded oxidoreductase protein and the *fucO*-encoded oxidoreductase protein for aerobic function provides two different examples for genetic adaptation. In the evolution of FucO, the first step was invariably the activation of the promoter (in 10/10 independent selections), followed by mutations in the coding region that conferred resistance to MCO (14). By contrast, in the evolution of AdhE, the first and second steps were invariably mutations in the coding region. The two biased modes of adaptation may illustrate how preexisting genetic, physiological, and biochemical contexts can predestine the channels for future evolution. The two examples also illustrate the versatility of the bacterial genome: when one evolutionary pathway is blocked, alternative routes are available.

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Addendum—Since the submission of this report, we found that preadaptation of wild-type cells on acetate as the sole source of carbon and energy allowed the appearance of colonies on solid minimal ethanol medium at a frequency of about 10^{-9}.

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Evolution of the *adhE* Gene Product of*Escherichia coli* from a Functional Reductase to a Dehydrogenase: GENETIC AND BIOCHEMICAL STUDIES OF THE MUTANT PROTEINS

Jorge Membrillo-Hernández, Pedro Echave, Elisa Cabiscol, Jordi Tamarit, Joaquim Ros and Edmund C. C. Lin

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