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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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 NH2-terminal region of the AdhE protein is highly homologous to aldehyde:NAD⁺ oxidoreductases, whereas the COOH-terminal region is homologous to a family of Fe²⁺-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/E568K. Purified AdhEA267T and AdhEA267T/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 AdhEA267T/E568K was more thermal stable than AdhEA267T. Since both mutant enzymes exhibited similar kinetic properties, the second mutation probably conferred an increased growth rate on ethanol by stabilizing AdhEA267T.

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, CO₂, and H₂ (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):

**Reaction 1**

AdhE

\[ \text{CH}_3\text{CO-S-CoA} + \text{NADH} + \text{H}^+ \rightarrow \text{CH}_3\text{CHO} + \text{CoA} + \text{NAD}^+ \]

**Reaction 2**

AdhE

\[ \text{CH}_3\text{CHO} + \text{NADH} + \text{H}^+ \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{NAD}^+ \]

AdhE appears to be the evolutionary product of a gene fusion. The NH2-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 Fe²⁺-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) cycle. In this disabling process, the amino acid chains of AdhE are thought to be covalently attacked by the highly reactive hydroxy radicals locally generated by the Fe²⁺ 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 Fe²⁺-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

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

**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-b-D-galactopyranoside.**

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**From the ‡Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115 and **Departament de Ciències Mèdiques Básiques, Facultat de Medicina, Universitat de Lleida, 25198 Lleida, Spain.
ceptively to MCO during aerobic metabolism. We have previously characterized E. coli mutants that acquired aerobic growth ability on 1,1,2-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 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 \( \mu \)M MOPS and 0.2% glucose was adjusted to pH 7.4 (LB-glucose medium). Minimal medium was prepared as described previously (16) and 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 by the Bradford method (22), using bovine serum albumin as standard.

Strains, plasmid, or phage | Relevant genotype | Source or Ref.
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CAG12169 | zci-506·Tn10 | M. Berlyn
DC272 | fadR mel tyr T adhC81 | 8
ECL3999 | MC4100 but adhE::kan | 30
ECL4000 | MC4100 (pAdhE-lacZ) | 30
ECL4060 | ECL4000 but adhC81 | ECL4000XP1(DC272)
ECL4063 | E399 attA::adhE\textsubscript{PJC} | This study
ECL4064 | E399 attA::adhE\textsubscript{PJC} | This study
ECL4065 | E399 attA::adhE\textsubscript{PJC} | This study
ECL4066 | E399 attA::adhE\textsubscript{PJC} | 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 | ΔargF- lacU189 rpc5150 araD189 relA1 thi-501 doaC1 ptsF25 | M. Casadaban
Plasmids
PJMADH1 | pBR322adhE\textsuperscript{*} from MC4100 | 30
PJMADH4 | pBR322adhE\textsubscript{PJC} | This study
PJMADH5 | pBR322adhE\textsubscript{PJC} | This study
PJMADH6 | pBR322adhE\textsubscript{PJC} | This study
pRS415 | Ap\textsuperscript{r} operon fusion vector | 25
Bacteriophages
ARS45 | Specialized phage for recombination with pRS vectors | 25
ADH Hop656 | (adhE-lacZ) Comprising up to 656 base pairs of the promoter region of adhE from MC4100 | 26

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 \( \times \) 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 \( \times \) 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. The 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 KC1, 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 \texttt{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 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 \( \mu \)M FeCl\(_3\) (23).
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 P1vir-mediated transduction (20). Standard methods were used for restriction endonuclease digestion and ligation of DNA (24, 25). Plasmid DNA was isolated by 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 Stratagen (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'-ATCACAAGTCATGATTGTAAGCAGTACGACGCT-3'), A3 (5'-GCCAATCTGACCGTGGATATCAGC-3'), B5 (5'-GGTATAGCCTGACGCTGGA-3'), B3 (5'-GCCCATCATATCATTTGGG-3'), C5 (5'-CTGCTCAAGACCCAGCAAAACCATGAGG-3'), D5 (5'-CTGCTGATGACCGCGG-3'), and D3 (5'-GAAGGGCTCGTTATGTTA-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 (Stratagen). Primers T5 (5'-TGCAAGCGATGCTTTCCACACCGGCGC-3') and T3 (5'-GCCCGCCTGGGTTCTGCTGAAATCCTGACG-3') were used to introduce the Ala267→Thr mutation in pJMADH4. Primers K5 (5'-GGGAACTCTCCCCGGGTCGTCGAGG-3') and K3 (5'-CGCGCGCGGCCTGGGTCGTCGAGG-3') were used to introduce the Lys568→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 at the CORE facility at Harvard Medical School.

**Insertion of Wild-type and Mutant adhE Alleles into Host Chromosome via λ Vectors**—The plasmids pJMADH1, pJMADH4, pJMADH5, and pJMADH6 were digested with restriction enzymes BamHI and EcoRI 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 (Stratagen, 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<sup>+</sup> ♀[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<sup>4</sup> 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<sup>+</sup> phenotype. Sixteen independent ethanol<sup>+</sup> 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 phase P1 lysate was then prepared from each of the mutants to transduce strain ECL4000 and selected for aerobic growth on ethanol. One ethanol<sup>+</sup> 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 of line of selection was then plated again on ethanol agar, and one colony that clearly exhibited an increased growth rate was isolated. A phase 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-positively 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 *Aeroglobin aerogenes*

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**Table II**

| Strain | Ethanol dehydrogenase activity<sup>a</sup> | β-Galactosidase activity<sup>b</sup> |
|--------|---------------------------------|---------------------------------|
|        | Glucose + O<sub>2</sub> | Glucose − O<sub>2</sub> | Ethanol + O<sub>2</sub> | Glucose + O<sub>2</sub> | Glucose − O<sub>2</sub> | Ethanol + O<sub>2</sub> |
| ECL4000 | 0.98 ± 0.04 | 0.95 ± 0.04 | 0.78 ± 0.10 | 970 ± 50 | 9700 ± 240 | 540 ± 40 |
| JE46    | 0.94 ± 0.10 | 1.61 ± 0.20 | 0.89 ± 0.10 | 580 ± 30 | 7000 ± 160 | 540 ± 40 |
| JE52    | 0.61 ± 0.10 | 1.98 ± 0.30 | 0.78 ± 0.10 | 520 ± 40 | 5300 ± 130 | 500 ± 60 |

<sup>a</sup> AdhE-specific activity is expressed as units/mg total protein.

<sup>b</sup> LacZ activity is expressed in Miller units (20).

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Evolution of a Complex Oxidoreductase of *E. coli* 33871
Evolution of a Complex Oxidoreductase of E. coli

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 same substitution found in strain JE46, plus an A to G transition at position 799 from the A of the initiation codon ATG. This change converts amino acid residue 568 that of DC48. It was suggested on the basis of genetic analysis that the mutation in DC81 altered a transcriptional site of the 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<sup>A267T</sup> and adhE<sup>A267T/E568K</sup> Mutant Alleles—To demonstrate that the mutations identified in strains JE46 (adhE<sup>A267T</sup>) and JE52 (adhE<sup>A267T/E568K</sup>) 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) showed aerobic and anaerobic levels of AdhE activity indistinguishable from those of the wild-type strains MC4100 and ECL4000. When strain ECL4064 (adhE::kan, λatt<sup>adhE<sup>EN677</sup></sup>) 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 ECLA066 (adhE<sup>A267T/E568K</sup>) 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, DC51, that acquired the ability to use ethanol as a substitute of acetate. Strain DC51 also grew on ethanol as sole carbon and energy source. When grown aerobically on glucose and acetate, DC51 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 DC51 altered a transcriptional site of the adhE 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<sup>+</sup> transductant of strain DC51 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

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 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.

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 P<sub>1</sub>vir lysate from each of the mutants to transduce strain CAG12169 that bears aceF<sup>+</sup>:Tn<sub>10</sub> insertion at min 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 799 from the A of the initiation codon ATG. This change converts 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.

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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

Kinetic Analysis of Purified AdhE, AdhEA267T, and AdhEA267T/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 AdhEA267T or AdhEA267T/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 AdhEA267T or AdhEA267T/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 AdhEA267T and AdhEA267T/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 *Clostridium 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 one 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
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 \textit{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 \textit{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.\footnote{J. Membrillo-Hernández, unpublished observations.}

The striking increase in the specific acetaldehyde dehydrogenase activity relative to the specific ethanol dehydrogenase activity of AdhE 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\textsuperscript{A267T/E568K} exhibits
ited kinetic properties indistinguishable from those of AdhE<sub>A267T</sub> 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<sub>A267T</sub> (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 mutations conferring only modest improvements failed to be efficacious of the novel function of AdhE that the MCO-resistant medium at a frequency of about 10<sup>-3</sup>.

Addendum—Since the submission of this report, we found that pre-adaptation 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<sup>-3</sup>.

### REFERENCES

1. Wood, W. A. (1961) in The Bacteria (Gunsalus, I. C., and Stainer, R. Y., eds) Academic Press, New York
2. Clark, D. P. (1989) FEMS Microbiol. Lett. 65, 223–234
3. Goodlove, P. E., Cunningham, P. R., Parker, J., and Clark, D. P. (1989) Gene (Amst.) 85, 209–294
4. Kessler, D., Herth, W., and Knappe, J. (1992) J. Biol. Chem. 267, 18073–18079
5. Cunningham, P. R., and Clark, D. P. (1986) Mol. Gen. Genet. 205, 487–493
6. Clark, D. P., and Crenan, J. E., Jr. (1980) J. Bacteriol. 144, 179–184
7. Chen, Y. M., and Lin, E. C. C. (1991) J. Bacteriol. 174, 8099–8103
8. Leonardo, M. R., Cunningham, P. R., and Clark, D. P. (1993) J. Bacteriol. 175, 870–878
9. Cabiscol, E., Aguilar, J., and Ros, J. (1994) J. Biol. Chem. 269, 6592–6597
10. Tamarit, J., Cabiscol, E., and Ros, J. (1998) J. Biol. Chem. 273, 3027–3032
11. Cocks, G. T., Aguilar, J., and Lin, E. C. C. (1974) J. Bacteriol. 118, 83–88
12. Srivastava, S., Wu, T. T., Chused, T. M., and Lin, E. C. C. (1969) J. Bacteriol. 98, 87–95
13. Conway, T. G., and Ingram, L. O. (1989) J. Bacteriol. 171, 3754–3759
14. Lu, Z., Cabiscol, E., Obradors, N., Tamarit, J., Ros, J., Aguilar, J., and Lin, E. C. C. (1998) J. Biol. Chem. 273, 8308–8316
15. Chen, Y. M., Lu, Z., and Lin, E. C. C. (1989) J. Bacteriol. 171, 6097–6105
16. Boronat, A., and Aguilar, J. (1981) Biochim. Biophys. Acta 672, 98–107
17. Boronat, A., and Aguilar, J. (1979) J. Bacteriol. 140, 320–326
18. Gupta, S., Mat-Jan, F., Latigi, M., and Clark, D. P. (2000) FEMS Microbiol. Lett. 182, 51–55
19. Racker, E. (1955) Methods Enzymol. 1, 500–503
20. Miller, J. H. (1972) A Short Course in Bacterial Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Laemli, U. K. (1970) Nature 227, 680–685
22. Bradford, M. M. (1976) Anal. Biochem. 72, 134–139
23. Levine, R. L. (1994) Methods Enzymol. 237, 370–376
24. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Simons, R. H., Cowen, L. E., and Kleckner, N. (1987) Gene (Amst.) 53, 85–96
26. Membrillo-Hernández, J., and Lin, E. C. C. (1999) J. Bacteriol. 181, 7571–7579
27. McPhedran, P., Somner, B., and Lin, E. C. C. (1991) J. Bacteriol. 81, 852–857
28. Leonardo, M. R., Dailly, Y., and Clark, D. P. (1996) J. Bacteriol. 178, 6013–6018
29. Berlyn, M. K. B., Low, B. K., and Rood, K. E. (1996) in Escherichia coli and Salmonella typhimurium Cellular and Molecular Biology (Neidhardt, F. C., ed) pp. 1715–1902, American Society for Microbiology, Washington, D. C.
30. Membrillo-Hernández, J., Kwon, O., De Wulf, P., Finkel, S., and Lin, E. C. C. (1999) J. Bacteriol. 181, 7390–7393
31. Clark, D. P., and Crenan, J. E. (1980) J. Bacteriol. 141, 177–183
32. Kessler, D., Leibrecht, I., and Knappe, J. (1991) FEBS Lett. 281, 59–63
33. Rosenthal, B., Mai, Z., Caplivski, D., Ghosh, S., de la Vega, H., Graf, T., and Samuelson, J. (1997) J. Bacteriol. 179, 3736–3736
34. Lurz, R., Mayer, F., and Gottschalk, G. (1979) Arch. Microbiol. 120, 255–262
35. Smith, I. T., and Kaplan, N. O. (1980) Arch. Biochem. Biophys. 203, 663–675

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