

Role of NAD in Regulating the *adhE* Gene of *Escherichia coli*

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The fermentative alcohol dehydrogenase of *Escherichia coli* is encoded by the *adhE* gene, which is induced under anaerobic conditions but repressed in air. Previous work suggested that induction of *adhE* might depend on NADH levels. We therefore directly measured the NAD⁺ and NADH levels for cultures growing aerobically and anaerobically on a series of carbon sources whose metabolism generates different relative amounts of NADH. Expression of *adhE* was monitored both by assay of alcohol dehydrogenase activity and by expression of $\phi(adhE'-lacZ)$ gene fusions. The expression of the *adhE* gene correlated with the ratio of NADH to NAD⁺. The role of NADH in eliciting *adhE* induction was supported by a variety of treatments known to change the ratio of NADH to NAD⁺ or alter the total NAD⁺-plus-NADH pool. Blocking the electron transport chain, either by mutation or by chemical inhibitors, resulted in the artificial induction of the *adhE* gene under aerobic conditions. Conversely, limiting NAD synthesis, by introducing mutational blocks into the biosynthetic pathway for nicotinic acid, decreased the expression of *adhE* under anaerobic conditions. This, in turn, was reversed by supplementation with exogenous NAD or nicotinic acid. In merodiploid strains carrying deletion or insertion mutations abolishing the synthesis of AdhE protein, an *adhE-lacZ* fusion was expressed at nearly 10-fold the level observed in an *adhE*⁺ background. Introduction of mutant *adhE* alleles producing high levels of inactive AdhE protein gave results equivalent to those seen in absence of the AdhE protein. This finding implies that it is the buildup of NADH due to lack of enzyme activity, rather than the absence of the AdhE protein per se, which causes increased induction of the $\phi(adhE'-lacZ)$ fusion. Moreover, mutations giving elevated levels of active AdhE protein decreased the induction of the $\phi(adhE'-lacZ)$ fusion. This finding suggests that the enzymatic activity of the AdhE protein modulates the level of NADH under anaerobic conditions, thus indirectly regulating its own expression.

The cofactor NAD plays a key role in many biological oxidation-reduction reactions. The maintenance of bacterial metabolism depends on these redox reactions both for biosynthetic intermediates and for the reducing equivalents generated. Thus, any investigation of the regulation of the metabolism of facultative anaerobes must consider the potential role of this cofactor. In *Escherichia coli*, the glycolytic pathway and tricarboxylic acid cycle are the major source of metabolic intermediates and the reduced cofactor, NADH (30). Without NADH reoxidation, the NAD⁺ pool would be quickly depleted, halting cellular metabolism and growth. When oxygen is present, reducing equivalents from NADH are transferred to the electron transport chain (ETC), generating H₂O and a membrane potential which is used to synthesize ATP (31). This regenerates NAD⁺ for use in subsequent reactions. When enterobacteria grow anaerobically, an electron transport system is available only if alternate electron acceptors, such as nitrate, trimethylamine *N*-oxide, or fumarate, are present (20).

If alternative oxidants are absent, the bacterial cell must recycle the NADH some other way, i.e., by fermentation. This involves using NADH to reduce metabolic intermediates and excreting the end products into the growth medium. This process results in the loss of most of the energy available from the carbon source but allows the bacteria to grow, albeit at a reduced rate. *E. coli* ferments sugars and their derivatives to a mixture of products, including acetate, lactate, succinate, formate, and ethanol (7). Most of the enzymes involved in fer-

mentation are induced anaerobically and are present only at basal levels during aerobic growth or anaerobic respiration. The enzyme of interest here, the fermentative alcohol dehydrogenase (ADH), is induced anaerobically and repressed by both oxygen and nitrate (8). The AdhE protein encodes the two enzyme activities, coenzyme A (CoA)-linked acetaldehyde dehydrogenase (ACDH) and ADH, which convert acetyl-CoA to ethanol (8). Kessler et al. (21, 22) have shown that the AdhE protein also regulates the enzymatic activity of pyruvate formate lyase (PFL), which is involved at a key point of fermentative metabolism. These observations suggest that the AdhE protein may play an important but currently undefined role in anaerobic metabolism.

Our previous work using proton nuclear magnetic resonance spectroscopy demonstrated that the molar ratios of the fermentation products generated depended on the oxidation state of the initial carbon source (1). More reduced carbon sources produced higher levels of ethanol than the more oxidized carbon sources. This finding led us to endorse the concept of redox balance, whereby the amount of NADH produced matches the amount of NADH consumed in the formation of each fermentation product. The decision between alternative fermentation products therefore depends on the amount of NADH generated during catabolism of the initial substrate to pyruvate (7). Nuclear magnetic resonance analysis demonstrated that the fermentation of glucose (4 atoms of hydrogen generated per C₆) was balanced by the equimolar production of ethanol (4 atoms of hydrogen consumed) and acetate (0 atoms of hydrogen consumed) (hydrogen atoms never actually released as free entities) (1). Recent experiments using $\phi(adhE'-lacZ)$ fusions have provided further support for the redox balance model. We demonstrated that transcriptional expression of *adhE* increased in accordance with the reduced state of the initial carbon source (26). The substrates used were

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TABLE 1. Strains of *E. coli* used

Strain	Relevant genotype	Reference or source
DC271	<i>fadR mel tyrT</i>	8
DC272	<i>adhC81</i> of DC271	8
DC430	<i>adhC81 adhR430</i> of DC271	9
DC658	<i>zch::Tn10 Δtrp</i> of DC272	Laboratory collection
DC921	<i>ΔlacU169</i> of DC430	Laboratory collection
DC1263	<i>ubiF zbf::Tn10</i>	Laboratory collection
DW114	<i>galE cydB::mini-Tn10</i>	37
GV102	<i>Δcyo::Tn5</i>	R. Gennis
JK599	<i>adhE::Kan</i>	14
LEO128	$\phi(adhE-lacZ)$ <i>ΔadhCE</i>	26
LEO204	$\phi(adhE-lacZ)$ <i>adhE⁺</i>	26
LEO205	$\phi(adhE-lacZ)$ <i>adhC</i>	26
LEO206	$\phi(adhE-lacZ)$ <i>adhC adhR</i>	DC921 × λ MRL1
LEO218	<i>cydB::mini-Tn10</i> of LEO204	LEO204 × P1(DW114)
LEO330	<i>pheA::Tn10</i> of LEO204	LEO204 × P1(NK6024)
LEO331	<i>nadA::Tn10</i> of LEO204	LEO204 × P1(NK6033)
LEO333	<i>adhE::Kan</i> of LEO204	LEO204 × P1(JK599)
LEO341	<i>Δcyo::Tn5</i> of LEO204	LEO204 × P1(GV102)
LEO351	<i>nadB</i> of LEO330	LEO330 × P1(PA3306)
LEO358	<i>adhC81 zch::Tn10 Δtrp</i> of LEO204	LEO204 × P1(DC658)
LEO360	<i>ubiF zbf::Tn10</i> of LEO204	LEO204 × P1(DC1263)
LEO381	<i>ana-1 zch::Tn10</i> of LEO204	LEO204 × P1(PRC16)
LEO385	<i>adhC81 adhE436 zch::Tn10</i> of LEO204	LEO204 × P1(PRC500)
LEO387	<i>adhC438</i> of LEO358	LEO358 × P1(PRC438)
LEO389	<i>cydB::mini-Tn10</i> of LEO341	LEO341 × P1(DW114)
NK6024	<i>pheA::Tn10</i>	N. Kleckner
NK6033	<i>nadA::Tn10</i>	N. Kleckner
PA3306	<i>pur1 nadB argH thi rpsL</i>	R. Lavallé
PRC16	<i>ana-1 zch::Tn10</i>	Laboratory collection
PRC438	<i>adhC438</i> of DC272	11
PRC500	<i>adhE436 zch::Tn10</i> of DC272	11

all glucose derivatives, differing only in the amounts of NADH generated during their catabolism. From these observations, we proposed that the NAD⁺/NADH ratio could be involved in controlling *adhE* expression. In this report, we further address the potential role of NADH in the regulation of *adhE* expression.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and strain construction. All bacteria used were strains of *Escherichia coli* K-12 and are described in Table 1. Rich broth contained (per liter) tryptone (10 g), NaCl (5 g), and yeast extract (1 g). Minimal medium M9 (27) or E (34) was supplemented with carbon sources at 0.4% (wt/vol) and, where appropriate, with amino acids (50 mg/liter). Vitamins, NAD analogs, and inhibitors of the ETC were added as indicated. PPPS medium contained (per liter) Bacto Peptone (17 g; Difco Laboratories, Detroit, Mich.), Difco Proteose Peptone No. 3 (3 g), and NaCl (5 g). Solid media contained 1.5% (wt/vol) Difco Bacto Agar. All anaerobic growth media were supplemented with trace metals as described previously (39). Growth was monitored turbidometrically, using a Klett-Summerson colorimeter equipped with a green (540-nm) filter. Anaerobic growth was performed in anaerobic jars (Oxoid Ltd., London, England) under an atmosphere of H₂ and CO₂ generated by Oxoid gas-generating kits. Resazurin indicators (Oxoid) were used to ensure anaerobic conditions. Anaerobic liquid cultures were grown without agitation in tubes inside the anaerobic jars or in milk dilution bottles filled to the top before sealing.

Gene fusion strains were constructed by lysogenizing appropriate host strains with λ MRL1, carrying the $\phi(adhE-lacZ)$ fusion, as described previously (26). Transductants using P1 *vir* were performed essentially as described by Miller (29). Transductants were selected on medium E containing glucose and 0.1% casein hydrolysate. Tetracycline was used at 10 mg/liter to select for the presence of Tn10. Kanamycin was used at 30 mg/liter in plates containing succinate and 0.1% casein hydrolysate.

Enzyme analysis and protein electrophoresis. Soluble cell extracts for ADH assays and protein analysis were made by breaking the bacteria in a French pressure cell (Aminco, Silver Spring, Md.) and then subjecting them to ultracentrifugation (8). Protein production was screened by sodium dodecyl sulfate (SDS)-polyacrylamide gel analysis (23). β -Galactosidase was assayed as described previously (39) except that cultures were grown to mid-exponential phase (approximately 5×10^8 cells/ml) before assay in a variety of media under both aerobic and anaerobic conditions. β -Galactosidase activity was measured as micromoles of *o*-nitrophenylgalactoside (ONPG) hydrolyzed per 10⁹ cells per hour at 37°C. ADH was assayed by observing the reduction of NAD⁺ to NADH at 340 nm (32). A unit of enzyme activity is defined as a nanomole of NADH produced per minute, with a limit of detection for unpurified cell lysate of approximately 0.4 U/mg of protein (8).

NAD-NADH pool isolation and NAD cycling assay. Ten-milliliter samples were removed from aerobic and anaerobic cultures, and the dinucleotides were extracted by a modification of the protocol described by Heber and Santarius (18). Duplicate 1.0-ml samples were placed in Eppendorf tubes and centrifuged at 15,000 × *g* for 1 min. After removal of the supernatant, the pellets were immediately frozen in a dry ice-ethanol bath. Then 250 μ l of either 0.2 M HCl (for NAD⁺ extraction) or 0.2 M NaOH (for NADH extraction) was added to the frozen pellets. Specific dinucleotides were extracted by placing the samples in a 100°C sand bath for 10 min and then subjecting them to centrifugation at 5,000 × *g* for 5 min to remove cellular debris. The dinucleotide-containing supernatants were transferred to fresh tubes and stored on ice until needed.

Assays of the extracts containing specific dinucleotide species were performed in triplicate by the recycling assay of Bernofsky and Swan (3). The assay reagent contains 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), phenazine ethosulfate (PES), ethanol, and yeast ADHII. The yeast ADHII converts NAD⁺ to NADH, utilizing ethanol. Briefly, NADH reduces PES, regenerating NAD⁺ for the next cycle. The PES is then oxidized by passing its reducing equivalents to MTT. The assay monitored the reduction of MTT at 570 nm in a Perkin-Elmer 552A UV/VIS spectrophotometer (Perkin-Elmer Cetus, St. Louis, Mo.). The reaction mixture contained the following: 100 μ l of 1.0 M Bicine (pH 8.0); 250 μ l of extract; 250 μ l of a neutralizing acid (0.1 M HCl for NADH) or alkali (0.1 M NaOH for NAD⁺); 100 μ l of 16.6 mM PES; 100 μ l of 4.2 mM MTT; 100 μ l of absolute ethanol; and 100 μ l of 40 mM EDTA (pH 8.0), which was preincubated for 3 min in a 1-ml cuvette. Twenty microliters of yeast ADHII (500 U/ml) in 0.1 M Bicine (pH 8.0) buffer was added to the reaction mixture to begin the assay. Increase in *A*₅₇₀ was recorded for 10 min. The rate of MTT reduction is proportional to the concentration of coenzyme (3). Coenzyme standards from 1.5 to 0.02 mM were used to calibrate the assay.

RESULTS

Measurement of NAD level and correlation with expression of the *adhE* gene. Previous work suggested that a change in the NAD⁺/NADH ratio might be a potential signal for the induction of *adhE* as observed with $\phi(adhE'-lacZ)$ fusions (26). The NAD⁺/NADH ratios for several different enterobacteria have been determined, using glucose as the growth substrate (16, 17, 38). We wanted to ascertain whether the ratio would differ in cultures grown with glucose derivatives of different oxidation

TABLE 2. NAD⁺/NADH ratios and expression of the *adhE* gene

Carbon source ^a	Aeration condition	Concn ^b		Ratio, [NAD ⁺]/[NADH]	<i>adhE</i> expression	
		NAD ⁺	NADH		ADH activity ^c	β -Gal activity ^d
Sorbitol	Aerobic	9.60	0.98	9.8	0.4	10,400
	Anaerobic	1.26	0.41	3.1	4.7	69,700
Glucose	Aerobic	2.56	0.24	10.6	0.3	7,100
	Anaerobic	1.03	0.23	4.5	3.0	45,000
Glucuronate	Aerobic	2.33	0.24	9.9	0.2	7,100
	Anaerobic	1.26	0.13	9.9	1.3	18,400

^a Strain DC271 was cultured in M9 plus proteose peptone supplemented with the indicated sugar derivatives (0.4% [wt/vol]).

^b Assayed as described in Materials and Methods and expressed as micromoles per milliliter of culture (adjusted to 5×10^8 cells per ml). The values shown are means of triplicate assays.

^c Expressed as nanomoles of NADH produced per minute per milligram of protein. ADH assays were performed with strain DC271.

^d β -Galactosidase (β -Gal) data for strain LEO204, which carries the $\phi(adhE'-lacZ)$ fusion in the DC271 background, are from reference 26. Values are expressed as micromoles of ONPG cleaved per hour per 10⁹ cells.

TABLE 3. Effects of nicotinic acid on $\phi(adhE'-lacZ)$ expression in strain LEO204

Growth condition ^a	Addition	β -Galactosidase sp act ^b		Ratio, anaerobic/aerobic
		Aerobic	Anaerobic	
-Glucose	Nicotinic acid (%)			
	None	4,600	30,000	6.5
	0.1	5,000	35,000	7.0
	0.2	5,300	36,000	6.8
	0.3	6,600	53,000	8.0
	0.4	6,200	68,000	11.0
+Glucose	0.5	5,300	62,000	11.7
	None	6,900	89,000	12.9
	0.1	9,200	91,000	9.9
	0.2	9,400	96,000	10.2
	0.3	9,900	168,000	17.0
	0.4	6,700	207,000	30.9
	0.5	7,800	203,000	26.0
	1 mM NAD	6,800	130,000	19.1
	1 mM NADH	7,400	95,000	12.8

^a Strain LEO204, carrying the $\phi(adhE'-lacZ)$ protein fusion, was grown in PPPS with or without glucose and supplemented with nicotinic acid as indicated.

^b Expressed as micromoles of ONPG cleaved per hour per 10^9 cells. The values shown are means of duplicate assays.

states. The NAD⁺ and NADH pools were extracted from cells grown both aerobically and anaerobically in M9 minimal medium plus sorbitol (3 NADH/C₆), glucose (2 NADH/C₆), or glucuronate (0 NADH/C₆). The concentration of the steady-state NAD⁺-plus-NADH pool from each sample was determined through the cycling assay of Bernofsky and Swan (3), and the NAD⁺/NADH ratio was calculated for each growth condition. As shown in Table 2, under aerobic culture conditions, these ratios (approximately 10:1) were essentially the same for all carbon sources. However, marked differences were observed between the various anaerobic cultures. The NAD⁺/NADH ratio was lowest in the sorbitol-grown cells (3.1:1), intermediate in glucose-grown cells (4.5:1), and in cells grown with glucuronate, almost as high as for aerobic cells (9.8:1). Moreover, the levels of both NAD⁺ and NADH were approximately fourfold higher for cells grown aerobically on sorbitol. The expression of *adhE*, as measured both by use of $\phi(adhE'-lacZ)$ expression and by assaying ADH activity, is also shown in Table 2. These observations are consistent with the hypothesis that the induction of the *adhE* gene is dependent on the NAD⁺/NADH ratio.

Manipulation of NAD metabolism and effect on expression of the *adhE* gene. The addition of nicotinic acid to culture medium has been shown to raise the overall level of NAD⁺ plus NADH in cells up to fourfold (27). To monitor the effects on $\phi(adhE'-lacZ)$ expression, excess nicotinic acid (0.1 to 0.5%, final concentration) was added to cultures of the fusion strain LEO204 growing in PPPS medium with or without glucose. PPPS is a rich medium which allows moderate anaerobic growth even in the absence of an exogenously added fermentable carbon source. As illustrated in Table 3, anaerobic induction of $\phi(adhE'-lacZ)$ increased with the concentration of nicotinic acid added. When glucose was present, both the absolute levels of $\phi(adhE'-lacZ)$ expression and the relative increases noted upon adding nicotinic acid were more marked (Table 3). Although NAD is not taken up intact by *E. coli*, the addition of 1 mM NAD⁺ produced a similar effect, presumably as a result of uptake of nicotinamide released by hydrolysis in the periplasmic space. In contrast, adding NADH had no effect,

TABLE 4. Effects of *nad* mutations on $\phi(adhE'-lacZ)$ expression

Strain ^a	Nicotinic acid (0.1%)	β -Galactosidase sp. act ^b		Ratio, anaerobic/aerobic
		Aerobic	Anaerobic	
LEO204 (wild type)	No	8,250	81,100	9.8
LEO331 (<i>nadA</i>)	No	9,100	20,600	2.3
	Yes	8,300	62,500	7.5
LEO351 (<i>nadB</i>)	No	9,500	17,650	1.9
	Yes	9,400	65,500	7.0

^a All strains carried the $\phi(adhE'-lacZ)$ protein fusion and were grown in PPPS plus 0.4% (wt/vol) glucose.

^b Expressed as micromoles of ONPG cleaved per hour per 10^9 cells. The values shown are the means of duplicate assays.

presumably as a result of the instability of this compound (Table 3).

Given the positive effect of adding nicotinic acid on $\phi(adhE'-lacZ)$ expression, it follows that mutations affecting NAD biosynthesis should lower the anaerobic induction of an $\phi(adhE'-lacZ)$ fusion. To test this inference, mutations in *nadB* (aspartate oxidase) and *nadA* (quinolinate synthetase A) were transduced into LEO204. When assayed for $\phi(adhE'-lacZ)$ expression by β -galactosidase activity (Table 4), both *nad* derivatives showed an induction ratio of only 2-fold (compared with 10-fold for the parental strain). The anaerobic induction of $\phi(adhE'-lacZ)$ was not completely abolished, possibly because of the trace amount of nicotinic acid in the PPPS medium (which is necessary to allow growth of the mutants). When excess nicotinic acid was added to the culture medium, it restored the anaerobic inducibility (Table 4). This finding accords with the idea that NADH levels affect the induction of the *adhE* gene.

Effects of ETC mutants and inhibitors on $\phi(adhE'-lacZ)$ expression. With the apparent response of $\phi(adhE'-lacZ)$ to NADH levels, it would seem logical that blocking the aerobic ETC would lead to artificial induction of *adhE* expression even in the presence of oxygen, as a result of the cells' inability to oxidize NADH. Strain LEO204 was transduced with P1 grown on strains carrying mutations affecting various components of the ETC. As seen in Table 5, derivatives of LEO204 carrying defects in *cydB* (LEO218) or *cyo* (LEO341) showed a slight (two- to threefold) increase in aerobic expression of the $\phi(adhE'-lacZ)$ fusion relative to their parent. This small effect with single cytochrome mutants is not surprising since *cyo* can be used as an alternative route when *cyd* is blocked, and vice versa (31), whereas a *cyo cyd* double mutant should completely block the ETC. Consistent with this observation, the *cyo cydB* double-mutant strain (LEO389) showed an almost 10-fold increase of $\phi(adhE'-lacZ)$ expression aerobically compared with

TABLE 5. Effects of respiratory chain mutations on $\phi(adhE'-lacZ)$ expression

Strain ^a	Mutation(s)	β -Galactosidase sp act ^b	
		Aerobic	Anaerobic
LEO204	None (wild type)	8,300	83,000
LEO218	<i>cydB</i>	13,000	109,000
LEO341	<i>cyo</i>	24,200	101,000
LEO389	<i>cyo, cydB</i>	73,200	124,000
LEO360	<i>ubiF</i>	30,800	81,000

^a All cultures were grown in PPPS supplemented with 0.4% (wt/vol) glucose.

^b Expressed as micromoles of ONPG cleaved per hour per 10^9 cells. The values presented are the means of duplicate assays.

TABLE 6. Effects of respiratory inhibitors and NAD analogs on $\phi(adhE'-lacZ)$ expression^a

Inhibitor or analog ^b (concn [mM])	β -Galactosidase sp act ^c	Induction (fold)
None	7,800	1.0
Cyanide (0.5)	52,700	6.8
Arsenite (0.5)	26,700	3.4
MICA (0.2)	24,500	3.2
3-Pyridine sulfonic acid		
1	27,900	3.6
10	19,700	2.5
6-Amino nicotinic acid		
1	28,200	3.6
10	34,900	4.5
20	27,200	3.5
3-Acetyl pyridine		
1	22,900	3.0
10	23,200	3.0

^a Strain LEO204 was grown in PPPS plus glucose (0.4% [wt/vol]) aerobically.

^b Inhibitors and analogs were added to aerobic cultures grown to 3×10^8 cells per ml.

^c Expressed as micromoles of ONPG cleaved per hour per 10^9 cells. The values presented are the means of duplicate assays.

LEO204 (Table 5). In fact, there was a less than twofold difference between aerobic and anaerobic expression of $\phi(adhE'-lacZ)$ in the double cytochrome mutant (Table 5). A mutation in the *ubiF* gene, which is required for ubiquinone synthesis, caused a similar, though more moderate, increase in aerobic expression (Table 5, strain LEO360). This finding suggests that loss of respiratory chain function, most likely due to the resultant buildup of NADH, has a major effect on the induction of the *adhE* gene.

We also disrupted the ETC by addition of various inhibitors, including several NAD analogs, in the hope of artificially inducing aerobic $\phi(adhE'-lacZ)$ expression. Cultures of LEO204 were grown in PPPS medium supplemented with 0.4% glucose until they reached 2×10^8 to 3×10^8 cells/mL, at which point the ETC inhibitors were added. Cultures were incubated for an additional 2 h, and samples were removed for the assay of β -galactosidase (Table 6). Cyanide increased expression of the $\phi(adhE'-lacZ)$ fusion nearly sevenfold at a concentration allowing growth at approximately 75% of the normal rate. Arsenite and the chelator 5-methoxyindole-2-carboxylic acid (MICA) are both inhibitors of the pyruvate dehydrogenase complex, and both gave approximately threefold increases in expression (Table 6).

The NAD analogs 3-pyridine sulfonic acid, 6-amino nicotinic acid, and 3-acetyl pyridine have been shown to inhibit NADH-using enzymes such as ADH and the NADH dehydrogenases of the ETC (12) while inducing the genes for NAD biosynthesis (19). As seen in Table 6, the addition of these compounds gave a modest induction of the $\phi(adhE'-lacZ)$ fusion aerobically, with 10 mM 6-amino nicotinic acid providing the greatest induction (4.5-fold). Addition of higher levels led to decreased expression and cessation of growth, as a result of toxic effects of these NAD analogs.

Effects of AdhE levels on $\phi(adhE-lacZ)$ expression. In our previous work, we noted that $\phi(adhE'-lacZ)$ expression decreased as the production of AdhE protein increased (26). This finding suggested that the presence of the AdhE polypeptide, its enzymatic activities, or its catabolic products might play some role in *adhE* regulation. We have addressed this question by using mutants expressing various levels of both active and inactive AdhE protein. The mutations were cotransduced into

TABLE 7. Effect of absence or inactivity of the AdhE protein on $\phi(adhE'-lacZ)$ expression

Strain ^a	Mutation(s)	β -Galactosidase sp act ^b	
		Aerobic	Anaerobic
LEO128	$\Delta adhCE$	16,300	503,000
LEO333	<i>adhE::Kan</i>	15,500	444,000
LEO381 ^c	<i>ana (adhE)</i>	11,500	429,000
LEO385 ^d	<i>adhC81 adhE436</i>	11,700	261,000
LEO204	None (wild type)	11,700	81,000
LEO387 ^e	<i>adhC438</i>	9,600	73,000
LEO205	<i>adhC81</i>	10,000	42,000
LEO206	<i>adhC81 adhR430</i>	5,600	12,100

^a All strains contain the $\phi(adhE-lacZ)$ protein fusion and were grown in PPPS supplemented with 0.4% (wt/vol) glucose.

^b Expressed as micromoles of ONPG cleaved per hour per 10^9 cells. The values presented are the means of duplicate assays.

^c Carries a mutation originally designated *ana* and now known to be in *adhE*, which produces normal amounts of an AdhE protein which has no ACDH or ADH activity.

^d Carries a mutation in *adhE* which produces normal amounts of an AdhE protein which has no ACDH activity but retains full ADH activity.

^e Carries a reversion of the *adhC81* constitutive mutation, selected by resistance to chloroacetaldehyde.

the $\phi(adhE'-lacZ)$ fusion strain LEO204 by using a nearby insertion of *Tn10* (except for the *adhE::Kan* insertion, which was selected directly by kanamycin). Strains carrying these mutations were grown both aerobically and anaerobically in M9-glucose medium supplemented with proteose peptone. Equal amounts (100 μ g) of protein from each sample were run on SDS–10% polyacrylamide gels to monitor production of AdhE (24). AdhE is an unusually large protein (molecular weight, 97,000) and is clearly visible as a major band in anaerobic samples.

Deletion of the whole *adhE* gene results in a five- to sixfold increase in expression of the $\phi(adhE'-lacZ)$ fusion over equivalent strains carrying an *adhE*⁺ allele, as illustrated in Table 7 by strain LEO128 and its parent LEO204. The *adhE::Kan* insertion (strain LEO333) gave the same result as the deletion, demonstrating that this effect is due to elimination of the AdhE protein rather than the absence of sequences in the promoter region.

The *ana* mutation of Chippaux et al. (6) maps to the *adhE* locus (25) and results in loss of both ADH and PFL activity (4). Strains carrying the *ana(adhE)* mutation expressed the AdhE polypeptide at the same level as the wild type (24). Preliminary sequence data indicate no alterations in the upstream region of the *ana(adhE)* mutant (40). In light of the PFL deactivase activity demonstrated by the AdhE polypeptide (21, 22), it seems likely that the *ana(adhE)* mutation produces an AdhE protein with increased PFL deactivase but no ADH activity. In any case, strain LEO381, carrying the *ana* mutation, showed the same large increase in expression of the $\phi(adhE'-lacZ)$ fusion as deletion and insertion mutants which completely lack the AdhE protein (Table 7). Strains with the *adhE436* mutation also expressed the AdhE polypeptide at the parental level (24). This mutant version of AdhE retains full ADH activity but has lost 99% of its ACDH activity (11). Strain LEO385, carrying the *adhE436* mutation, also showed substantially increased expression of the $\phi(adhE'-lacZ)$ fusion, though not to as great an extent as in mutants completely lacking AdhE protein (Table 7). Unfortunately, no mutants lacking the ADH activity of AdhE but retaining the ACDH activity are available.

Strains carrying *adhC* constitutive promoter mutations produce over 10-fold the wild-type level of AdhE protein irrespec-

tive of whether they are grown aerobically or anaerobically (8). The addition of an *adhR* mutation increases AdhE synthesis another 10-fold over the level found in *adhC* strains (9). The presence of *adhC* alone (LEO205) or in combination with *adhR* (LEO206) decreases the level of expression of $\phi(adhE'-lacZ)$ by two- or sevenfold respectively (Table 7). The *adhC438* mutation is due to reversion of the *adhC81* promoter mutation rather than a defect in the *adhE* structural gene. Strains carrying the *adhC438* mutation have lost the aerobic expression of both ADH and ACDH activities typical of constitutive *adhC* mutants but express normal wild-type anaerobic levels of both ADH and ACDH, suggesting that the *adhC* mutation has reverted. They also show a protein synthesis pattern similar to those of wild-type strains (data not shown). The *adhC* revertant strain LEO387 regained the $\phi(adhE'-lacZ)$ induction level characteristic of the wild-type strain LEO204 (Table 7).

The presence of the AdhE protein itself did not appear to affect the regulation of the *adhE* gene. Rather, our results suggest that the ACDH activity of AdhE is critical in determining the level of *adhE* expression. (Note that if ACDH is not functioning, there will be no acetaldehyde to act as a substrate for the ADH reaction.) The greater the enzyme activity, the faster the recycling of NADH and the lower its steady-state concentration. Hence, these observations are all consistent with induction of *adhE* depending on the NADH level. An alternative possibility is that ethanol and acetaldehyde, the products of the two enzyme activities of AdhE, are involved in *adhE* regulation. To test this possibility, strain LEO204 was grown in PPPS medium under aerobic or anaerobic conditions with various concentrations of ethanol or acetaldehyde. Neither compound had any significant effect on the level of $\phi(adhE'-lacZ)$ expression (data not shown).

DISCUSSION

The fermentation pathway from acetyl-CoA to ethanol requires two sequential enzymatic activities: CoA-linked ACDH and ADH (7). In *E. coli*, both activities are functions of the *adhE* gene product (8) and are induced some 10-fold under anaerobic conditions (8). Both operon and protein fusions of *adhE* to the *lacZ* gene are induced anaerobically to the same extent, demonstrating that induction occurs at the level of transcription rather than translation or enzyme activation (26).

Growth on sugar derivatives of different oxidation states greatly affects expression of the *adhE* gene (26). Expression of *adhE*, whether measured by assaying ADH activity from an *adhE*⁺ gene or β -galactosidase from an $\phi(adhE'-lacZ)$ fusion, was highest when cells were grown with sorbitol. Such sugar alcohols are more reduced than glucose and so yield more NADH (7). In contrast, gluconic and glucuronic acids yield less NADH than glucose, and the anaerobic induction of *adhE* was largely abolished when these were growth substrates.

The relative levels of NADH and NAD⁺ have been measured directly in chemostat cultures of *Klebsiella aerogenes* by using fluorimetry. The NADH level increased when the oxygen supply was shut off and dropped when oxygenation was resumed (16, 17). The NADH/NAD⁺ ratio in *E. coli* was 0.29 under aerobic conditions, and 0.67 anaerobically, for cultures grown on glucose plus tryptone (38). Similar shifts were found for several other species of bacteria. Overall, the best estimate of the anaerobic increase in the NADH/NAD⁺ ratio would seem to be approximately twofold, during glucose metabolism (38).

Our direct analysis of the NADH/NAD⁺ ratios of *E. coli* DC271 grown aerobically and anaerobically with three different carbon sources (sorbitol, glucose, and glucuronate) agreed

with these estimates (Table 2). Moreover, the NADH/NAD⁺ ratios increased with the reduced state of the carbon source and correlated well with *adhE* expression. It is the NADH/NAD⁺ ratio, rather than the absolute level of NADH, which correlates best with *adhE* expression. These data concur with our earlier proton nuclear magnetic resonance analysis indicating that the proportion of ethanol in the fermentation mixture decreases as the oxidation state of the growth substrate increases (1).

Internal levels of NAD(H) can be modulated by adding excess nicotinic acid, which is converted to nicotinic acid mononucleotide (12). This can lead to a fourfold increase in internal levels of NAD(H) (27). Such augmentation of the NAD(H) pool resulted in a moderate increase in $\phi(adhE'-lacZ)$ expression (Table 3). Conversely, disruption of NAD biosynthesis by mutations in the *nadA* or *nadB* gene (encoding quinolate synthetase [34]) led to a marked decrease in $\phi(adhE'-lacZ)$ expression. This, in turn, could be counteracted by supplementation with nicotinic acid (Table 4). These observations again suggest that the level of NADH is important in regulating *adhE* expression.

Inhibiting the aerobic ETC, either by the inactivation of cytochrome genes or with cyanide, artificially induced $\phi(adhE'-lacZ)$ expression aerobically (Tables 5 and 6). Such a loss of respiratory chain function would prevent NADH reoxidation, and $\phi(adhE'-lacZ)$ expression would increase in response to the buildup of NADH. Agents which collapse the proton motive force but do not inhibit electron flow had no effect on *adhE* expression (data not shown). This finding implicates the buildup of NADH (or a related metabolite), rather than energy starvation, as the critical factor. In support of this interpretation, the addition of NAD analogs to aerobic cultures also led to elevated aerobic expression of $\phi(adhE'-lacZ)$ (Table 6). One so far unexplained observation is that two agents (arsenite and MICA) which primarily inhibit pyruvate dehydrogenase promoted a moderate aerobic expression of the $\phi(adhE'-lacZ)$ fusion.

Previous work showed that the level of AdhE protein greatly influenced the anaerobic induction of $\phi(adhE'-lacZ)$. The lower the level of AdhE, the greater the anaerobic induction of $\phi(adhE'-lacZ)$ (26). The dual ADH and ACDH activities of the AdhE protein provide the major route for recycling NADH during fermentation, and their absence would result in a decreased rate of NADH consumption. The opposite would occur in strains which overproduce AdhE (8). The simplest interpretation is that the lower the level of AdhE, the greater the buildup of NADH and the greater the induction of the *adhE* gene. Less likely is that the AdhE protein itself has a direct regulatory effect. To address this question, several point mutations in *adhE*, which result in the synthesis of AdhE proteins with little or no enzyme activity, were introduced into $\phi(adhE'-lacZ)$ strains. The expression of $\phi(adhE'-lacZ)$ correlated with the level of enzyme activity rather than the presence or absence of the AdhE protein (Table 7), supporting the contention that the expression of $\phi(adhE'-lacZ)$ correlates with the presumed levels of NADH.

This use of NADH levels as a regulatory signal is not unique. During fermentation in *Clostridium acetobutylicum*, the shift from acid to solvent production appears to depend on the level of NADH (2). As fermentation progresses, the level of NADH increases and induces the shift from acidogenesis to solventogenesis. When levels of NADH decrease as a result of solvent production, this organism shifts back to acidogenesis (15). The solventogenic enzymes are induced under high-NADH conditions, whereas several key glycolytic and acidogenic enzymes are repressed under these conditions (13, 35).

How NADH affects the induction of the *adhE* gene is still obscure. NADH might bind directly to a regulatory protein. Alternatively, NADH might reduce a prosthetic group on the regulatory protein, thus altering its activity. Mutations in the *adhR* gene result in greatly elevated levels of ADH in an *adhC* background (9) and prevent growth on acetate unless the culture is supplemented with nicotinic acid (10). The *adhR* gene seems a likely candidate for regulation of the *adhE* gene in response to NADH. Further investigation of the role of AdhR in regulating the *adhE* gene is obviously necessary.

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