Regulation of the *adhE* Gene, Which Encodes Ethanol Dehydrogenase in *Escherichia coli*

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The respiratory control of *adhE*, which encodes ethanol (alcohol) dehydrogenase in *Escherichia coli*, was examined at the transcriptional level by using various $\Phi(adhE'-lacZ)$ *adhE*⁺ merodiploid strains. Expression of the *adhE'-lacZ* operon fusion was increased about eightfold by anaerobic growth. Under anaerobic growth conditions, provision of nitrate lowered the level of expression. Nitrate repression was more severe than aerobic repression. From analyses of various mutants with mutations related to nitrate reduction, nitrate repression appears to result from two effects. (i) When nitrate was present, NarL, the positive regulator of the *nar* operon, exerted a direct repression on *adhE* expression, which was demonstrable even aerobically. (ii) The chemical reduction of nitrate exerted an indirect effect by altering the cellular redox potential.

The facultative anaerobe *Escherichia coli* carries out mixed-acid fermentation during anaerobic growth in the absence of exogenous electron acceptors. The fermentation products are formate, ethanol, acetate, D-lactate, succinate, CO₂, and H₂. Ethanol, a major product, is synthesized from acetyl coenzyme A by two consecutive NADH-dependent reductions catalyzed by a single protein with an acetaldehyde domain and an ethanol dehydrogenase domain. The *adhE* gene (min 27) encoding the protein was cloned, and an open reading frame comprising 891 codons was found (9, 20; for a review, see reference 5). Recently, it was discovered that the *adhE* protein was also responsible for the pyruvate-formate-lyase-deactivase activity (18).

In our early study of ethanol dehydrogenase in *Klebsiella pneumoniae*, the enzyme activity level was increased more than 10-fold by anaerobic growth with glycerol as the carbon and energy source. The addition of fumarate as an electron acceptor to the anaerobic culture decreased (the activity level by more than fourfold (24). The anaerobic growth effect on the level of ethanol dehydrogenase activity was also observed in *E. coli*; furthermore, nitrate decreased this activity level both aerobically and anaerobically (4). Since several NAD-linked fermentation enzymes are posttranslationally inactivated during aerobic metabolism (2, 15, 21), it is not clear to what extent the low activity level of ethanol dehydrogenase measured in aerobically grown cells reflects transcriptional control. The problem is further complicated by the presence of an alcohol dehydrogenase activity associated with aerobic metabolism (36). In the present study, we tried to characterize the transcriptional control of the *adhE* gene by using a merodiploid strain bearing the wild-type *adhE*⁺ and a copy of $\Phi(adhE'-lacZ)$ on the chromosome.

Cloning of the *adhE* gene and construction of the $\Phi(adhE'-lacZ)$ *adhE*⁺ merodiploid strain. An 8-kb *EcoRI*-*PvuII* fragment from λ 3D5 (19) DNA was cloned into the *EcoRI*-*EcoRV* sites of the plasmid vector pBluescript to construct pADH7 (Fig. 1). This plasmid contained *adhE* and an IS2 element upstream of the gene (35). To delete the IS2 element, which might cause instability of the plasmid (9), pADH8 was constructed by deleting the *MluI*-*XhoI* fragment

from pADH7. The *MluI* site is about 0.4 kb downstream of the 3' terminus of IS2 and about 0.9 kb upstream of the open reading frame of *adhE*, and the *XhoI* site is in the polylinker region on the vector. Thus, pADH8 should contain the entire *adhE* gene (Fig. 1). When pADH8 was transformed into strain MRi93, bearing a *pcnB* mutation that reduces the plasmid copy number four- to fivefold (22), the level of ethanol dehydrogenase activity of the transformants under both aerobic and anaerobic conditions was three to four times higher than that of the cells bearing the vector alone. Moreover, the enzyme activity level of anaerobically grown cells was severalfold higher than that of the aerobically grown cells (data not shown). Therefore, in pADH8, the 0.9-kb fragment upstream of the *adhE* open reading frame contained the regulatory region responsible for the respiratory transcriptional control.

To construct the *adhE'-lacZ* operon fusion, the 1.4-kb *MluI*-*BglII* fragment spanning about 0.9 kb upstream and 0.5 kb downstream of the initiation codon of *adhE* was cloned into the *SmaI*-*BamHI* sites of the fusion vector pRS415 (28). The constructed $\Phi(adhE'-lacZ)$ plasmid pADH102 was transferred into λ RS45 (28) by homologous recombination *in vivo*, and the resultant recombinant phage λ ADH12 was used to lysogenize appropriate *E. coli* hosts (Table 1) according to the procedures described by Simons et al. (28). Strain ECL1100 bearing *adhE*⁺ and $\Phi(adhE'-lacZ)$, identified as a single lysogen, was chosen for this study and used as the host to construct various mutants (Table 1) by P1vir transduction (25).

Aerobic and anaerobic expression of *adhE*. Cultures of wild-type strain MC4100 (*adhE*⁺) and its isogenic derivative ECL1100 [$\Phi(adhE'-lacZ)$ *adhE*⁺] grown under aerobic and anaerobic conditions were assayed for ethanol dehydrogenase and β -galactosidase activities (Table 2). In both strains, the activity level of ethanol dehydrogenase was six- to sevenfold higher in anaerobically grown cells than in aerobically grown cells. In strain ECL1100, the activity level of β -galactosidase was about eightfold higher in anaerobically grown cells than in aerobically grown cells. The respiratory growth condition thus exerts transcriptional control on *adhE*. Unexpectedly, the ethanol dehydrogenase activity levels varied almost in parallel with those of β -galactosidase. This might indicate that under our experimental conditions

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FIG. 1. Cloning of the *adhE* gene and construction of the Φ (*adhE'*-*lacZ*) plasmid pADH102. The solid lines represent inserted DNA fragments in the plasmid vectors. The number of kilobases on the top line corresponds to the kilobase pair of the chromosome on the map of Kohara et al. (19). Relevant restriction sites are indicated as follows: E, *EcoRI*; Pv, *PvuII*; Bg, *BglII*; P, *PstI*; H, *HindIII*; M, *MluI*; S, *SmaI*; X, *XhoI*; B, *BamHI*. M/X, the joining of blunt-ended *MluI* and *XhoI*; B/Bg, the joining of *BamHI* and *BglII*; S/M, the joining of *SmaI* and blunt-ended *MluI*. Genes are indicated as open bars. Arrows represent the direction of transcription.

the aerobic activity loss of the *adhE*-encoded fermentative ethanol dehydrogenase, observed in *K. pneumoniae* (15) and which we confirmed in *E. coli* (unpublished data), was counterbalanced by the increased synthesis of the aerobic alcohol dehydrogenase (36).

Anaerobic effects of exogenous electron acceptors on the

expression of *adhE*. Nitrate or fumarate respiration is known to decrease the anaerobic activity level of ethanol dehydrogenase (4, 24). To test whether the effect is primarily transcriptional, we assayed the activity level of ethanol dehydrogenase and β -galactosidase in strain ECL1100 grown anaerobically in the absence or presence of nitrate, trimeth-

TABLE 1. *Escherichia coli* K-12 strains, phages, and plasmids

Strain, phage, or plasmid	Relevant genotype	Source or reference
Strains		
MC4100	<i>F⁻ araD139 ΔlacU169 rpsL150 relA1 ffb-5301 deoC1 ptsF25</i>	M. J. Casadaban
RK5263	<i>narH200::Tn10 araD139 gyrA ΔlacU169 non rpsL</i>	33
RK5267	<i>narI204::Tn10 araD139 gyrA ΔlacU169 non rpsL</i>	33
RK5278	<i>narL215::Tn10 araD139 gyrA ΔlacU169 non rpsL</i>	33
LK3110	MC4100 <i>narX</i>	R. P. Gunsalus
IS4	MC4100 Δ <i>narXL</i>	R. P. Gunsalus
MRi93	<i>pcnB80 zad::Tn10</i>	22
ECL562	<i>frd-101::λp1(209) chlE103 zbi-624::Tn10</i>	12
ECL1100	MC4100 Φ (<i>adhE'</i> - <i>lacZ</i>) <i>adhE</i> ⁺	λ ADH12 $\times$ MC4100
ECL1101	As ECL1100, but <i>narH200::Tn10</i>	P1(RK5263) $\times$ ECL1100
ECL1102	As ECL1100, but <i>narI204::Tn10</i>	P1(RK5267) $\times$ ECL1100
ECL1103	As ECL1100, but <i>narL215::Tn10</i>	P1(RK5278) $\times$ ECL1100
ECL1104	As ECL1100, but <i>narX</i>	λ ADH12 $\times$ LK3110
ECL1105	As ECL1100, but Δ <i>narXL</i>	λ ADH12 $\times$ IS4
ECL1106	As ECL1100, but <i>chlE103 zbi-624::Tn10</i>	P1(ECL562) $\times$ ECL1100
ECL1107	As ECL1100, but Δ <i>narXL chlE103 zbi-624::Tn10</i>	P1(ECL562) $\times$ ECL1105
Phages		
λ 3D5		19
λ RS45	<i>lacZ' lacYA⁺ bla'</i>	28
λ ADH12	Φ (<i>adhE'</i> - <i>lacZ</i>) <i>lacYA⁺ bla'</i>	This study
Plasmids		
pRS415	<i>Ap^r lacZYA⁺</i>	28
pADH7	<i>Ap^r adhE⁺ IS2⁺</i>	This study
pADH8	<i>Ap^r adhE⁺</i>	This study
pADH102	<i>Ap^r Φ(<i>adhE'</i>-<i>lacZ</i>) lacYA⁺</i>	This study

TABLE 2. Aerobic and anaerobic expression of *adhE* in wild-type strain MC4100 and the merodiploid strain ECL1100 grown in LB medium^a

Strain	Genotype	Relative activity					
		Ethanol dehydrogenase ^b			β-Galactosidase ^c		
		−O ₂	+O ₂	−O ₂ /+O ₂ ratio	−O ₂	+O ₂	−O ₂ /+O ₂ ratio
MC4100	<i>adhE</i> ⁺	95	15	6.3	0	0	
ECL1100	Φ(<i>adhE'</i> - <i>lacZ</i>) <i>adhE</i> ⁺	100 ^d	15	6.7	100 ^e	12	8.3

^a LB medium contains 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.4% glucose, and 0.1 M 3-(*N*-morpholino) propanesulfonate (MOPS) at pH 7.6. Incubation of aerobic and anaerobic cultures and preparation of cell extracts were carried out as described previously (3). Potassium phosphate buffer (10 mM, pH 8.2) was used for suspension of the cells.

^b Ethanol dehydrogenase activity was assayed spectrophotometrically by the rate of NADH formation at 340 nm. The assay mixture (1 ml) consisted of 1.6 M ethanol, 0.3 M potassium carbonate buffer (pH 10.0), and 0.66 mM NAD (24).

^c β-Galactosidase was assayed according to the method of Miller (25), but the specific activity was expressed as micromoles per minute per milligram of protein.

^d The specific activity was 380 ± 40 nmol/min/mg of protein. The enzyme units given were the average of three experiments.

^e The specific activity was 21 ± 2 μmol/min/mg protein. The enzyme units given were the average of three experiments. When assayed in whole cells treated with sodium dodecyl sulfate and chloroform, the activity was about 7,000 Miller units. The λ lysogen bearing *lacZ* without the *adhE* promoter gave 5 U of activity.

ylamine-*N*-oxide (TMAO), and fumarate. Nitrate repressed the activity level of ethanol dehydrogenase and β-galactosidase 11- and 12-fold, respectively (Table 3). TMAO or fumarate reduced both activities about twofold (data not shown).

Effects of *nar* regulatory genes and nitrate reduction on the repression of *adhE*. Two different mechanisms have been recognized in anaerobic enzyme induction and repression by nitrate. The first involves a two-component regulatory system: *narX*, which encodes the sensor protein, and *narL*, which encodes the regulator protein (8, 17, 26, 34). In the presence of nitrate and molybdate, NarL becomes an activator for the *narGHJI* operon encoding the nitrate reductase complex but a repressor for other operons such as *frdABCD*, which encodes fumarate reductase (12, 13, 16, 30), the *dms* genes, which encode TMAO-dimethyl sulfoxide reductase (7), or the *adhE* gene (16). The second mechanism of nitrate regulation involves the chemical reduction of nitrate, which alters the cellular redox potential. Nitrate reduction can be catalyzed by two different enzyme complexes. The first is encoded by *narGHJI*, and the second is encoded by *narZ*, which is not under NarL control (1, 30). Since both enzymes require the molybdenum cofactor for activity, nitrate reduction can be completely prevented by a mutation in *chlE* which blocks the synthesis of the cofactor (31).

To study further the anaerobic nitrate repression of *adhE*, we compared the properties of strains bearing mutations in *narX*, *narL*, *narH*, *narI*, or *chlE* (Table 3). In strains ECL1104 (*narX*), ECL1103 (*narL*), and ECL1105 (*ΔnarXL*), the repression was relieved four- to fivefold. In contrast, the repression was only slightly relieved in strains ECL1101 (*narH*) and ECL1102 (*narI*). In strain ECL1106 (*chlE*), the repression was relieved about fivefold. In the *ΔnarXL chlE* double mutant ECL1107, the repression was completely abolished. It therefore appears that the nitrate repression observed in wild-type cells reflected the additive effects of NarL and the change in cellular redox potential caused by nitrate reduction. It might be noted that in the *narX*, *narL*, and *chlE* mutants, the anaerobic activity levels of ethanol dehydrogenase and β-galactosidase were both subnormal. Perhaps the decreased enzyme levels were associated with the inferior anaerobic growth of the mutants.

The direct role of NarL is supported by the observation that in strain ECL1100 nitrate exerted a slight but significant repression even aerobically, when no significant nitrate reductase activity would be expected. No aerobic nitrate repression was observed in the *narL* mutant ECL1103 (Table 3).

The strong degree of repression by nitrate ($E'^{\circ} = +430$ mV), exceeding that of O₂ ($E'^{\circ} = +820$ mV), would suggest

TABLE 3. Effects of *nar* and *chl* mutations on nitrate repression of *adhE* and Φ(*adhE'*-*lacZ*) expression

Strain	Relevant mutation	Growth condition ^a	Relative activity					
			Ethanol dehydrogenase			β-Galactosidase		
			−NO ₃	+NO ₃	−NO ₃ /+NO ₃ ratio	−NO ₃	+NO ₃	−NO ₃ /+NO ₃ ratio
ECL1100		Anaerobic	100 ^b	9	11	100 ^c	8.3	12
ECL1101	<i>narH</i> ::Tn10	Anaerobic	86	11	7.8	96	9.6	10
ECL1102	<i>narI</i> ::Tn10	Anaerobic	89	10	8.9	96	11	9
ECL1103	<i>narL</i> ::Tn10	Anaerobic	69	31	2.2	73	37	2
ECL1104	<i>narX</i>	Anaerobic	71	30	2.4	64	24	2.7
ECL1105	<i>ΔnarXL</i>	Anaerobic	80	31	2.6	86	40	2.2
ECL1106	<i>chlE</i>	Anaerobic	60	27	2.2	52	20	2.6
ECL1107	<i>ΔnarXL chlE</i>	Anaerobic	70	81	0.9	86	96	0.9
ECL1100		Aerobic	15	11	1.4	11	8	1.4
ECL1103	<i>narL</i> ::Tn10	Aerobic	8.6	11	0.8	10	11	0.9

^a The growth conditions were as given for Table 2. When it was used, nitrate was added at 40 mM.

^b The specific activity was 350 ± 40 nmol/min/mg of protein. The enzyme units given were the average of three experiments.

^c The specific activity was 22 ± 2 μmol/min/mg of protein. The enzyme units given were the average of three experiments.

that the transcriptional apparatus was responding to changes of internal redox potential and that O₂ was not a direct effector. A mutation in each of the four pleiotropic regulatory genes, *fnr* (29), *rpoN* (10), *arcA* (14), and *crp* (23), was tested for its effect on the regulation of *adhE* expression. No significant influence was observed (data not shown). The question remains whether there is a redox-sensing regulator protein that is responsible for regulating *adhE* expression. The same mechanism of redox repression might play a role in the aerobic repression of *fdhF*, which encodes formate dehydrogenase-H; *hycE* (*hyd-17*), which encodes hydrogenase 3 (32); *pfl*, which encodes pyruvate-formate lyase (27); and *glpA*, which encodes anaerobic glycerol-3-phosphate dehydrogenase (11). Two genes affecting the activity level of ethanol dehydrogenase, however, have been reported. A mutation in *adhR* at min 72 increased the activity level of ethanol dehydrogenase about 10-fold, and a mutation in *adhB* at min 28 decreased the anaerobic activity level of ethanol dehydrogenase by 90% (5, 6). The availability of the $\Phi(adhE'-lacZ)$ *adhE*⁺ merodiploid strain should be useful in the selection and screening of *trans*-acting mutants that escape respiratory control. The characterization of such mutants may help to clarify the mechanism of transcriptional regulation of *adhE*.

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