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Dihydrolipoamide Dehydrogenase Mutation Alters the NADH Sensitivity of Pyruvate Dehydrogenase Complex of *Escherichia coli* K-12[▽]

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Under anaerobic growth conditions, an active pyruvate dehydrogenase (PDH) is expected to create a redox imbalance in wild-type *Escherichia coli* due to increased production of NADH (>2 NADH molecules/glucose molecule) that could lead to growth inhibition. However, the additional NADH produced by PDH can be used for conversion of acetyl coenzyme A into reduced fermentation products, like alcohols, during metabolic engineering of the bacterium. *E. coli* mutants that produced ethanol as the main fermentation product were recently isolated as derivatives of an *ldhA pflB* double mutant. In all six mutants tested, the mutation was in the *lpd* gene encoding dihydrolipoamide dehydrogenase (LPD), a component of PDH. Three of the LPD mutants carried an H322Y mutation (*lpd102*), while the other mutants carried an E354K mutation (*lpd101*). Genetic and physiological analysis revealed that the mutation in either allele supported anaerobic growth and homoethanol fermentation in an *ldhA pflB* double mutant. Enzyme kinetic studies revealed that the LPD(E354K) enzyme was significantly less sensitive to NADH inhibition than the native LPD. This reduced NADH sensitivity of the mutated LPD was translated into lower sensitivity of the appropriate PDH complex to NADH inhibition. The mutated forms of the PDH had a 10-fold-higher K_i for NADH than the native PDH. The lower sensitivity of PDH to NADH inhibition apparently increased PDH activity in anaerobic *E. coli* cultures and created the new ethanologenic fermentation pathway in this bacterium. Analogous mutations in the LPD of other bacteria may also significantly influence the growth and physiology of the organisms in a similar fashion.

Escherichia coli, a facultative heterotroph, grows under aerobic and anaerobic conditions. During aerobic growth, this bacterium metabolizes glucose through the reactions of glycolysis, pyruvate dehydrogenase (PDH), and the tricarboxylic acid cycle. The NADH generated during these enzyme-catalyzed reactions is oxidized ultimately by oxygen. Under anaerobic conditions and in the absence of external electron acceptors, organic compounds generated from glucose during glycolysis serve as the electron acceptors to maintain the redox balance and continued growth of the bacterium. Due to the differences in electron acceptors between the two growth modes, the reported [NADH]/[NAD^+] ratio of an anaerobic cell is severalfold higher (about 0.75) than that of an aerobic cell (about 0.03) (13, 33).

The PDH complex that connects glycolysis and tricarboxylic acid cycle enzymes is composed of multiple subunits of three enzymes, pyruvate decarboxylase (dehydrogenase; enzyme 1 [E1]; EC 1.2.4.1), dihydrolipoamide acetyltransferase (enzyme 2 [E2]; EC 2.3.1.12), and dihydrolipoamide dehydrogenase (LPD) (enzyme 3 [E3]; EC 1.8.1.4) (14). NADH, a product of the PDH reaction, is a competitive inhibitor of the PDH complex (15, 30, 31). The NADH sensitivity of the PDH complex has been demonstrated to reside in LPD, the enzyme that interacts with NAD^+ as a substrate (29, 30, 38). Although PDH is critical for aerobic growth of the bacterium, this activity was also detectable in cell extracts of *E. coli* grown under anaerobic conditions (13, 32, 33). However, based on the product profile, the PDH activity in vivo in anaerobic *E. coli* cultures is either very low or undetectable (33).

In an anaerobically growing *E. coli* strain lacking PDH activity, pyruvate is metabolized by an alternative enzyme, pyruvate-formate lyase, to acetyl coenzyme A (acetyl-CoA) with conservation of the reductant as formate (9). Formate is ultimately removed as H_2 and CO_2 without influencing the [NADH]/[NAD^+] ratio of the cell (28). In order to maintain the redox balance, the NADH generated during the oxidation of glyceraldehyde-3-phosphate in the glycolysis pathway is oxidized using acetyl-CoA as the electron acceptor, with the production of ethanol (9). However, reduction of acetyl-CoA to ethanol by alcohol dehydrogenase requires two NADH molecules for each acetyl-CoA molecule, a demand that is not met by fermenting *E. coli*. Due to this constraint, the fermentation profile of a growing *E. coli* strain includes equimolar quantities of ethanol and acetate.

We recently isolated and described *E. coli* mutants that produced ethanol as the main fermentation product (19). The mutation in one of these mutants was mapped in the genes of the *pdh* locus (*pdhR*, *aceEF*, and *lpd*). Based on the phenotype and genetic analysis, it was inferred that PDH, the enzyme that is normally inactive in an anaerobic *E. coli* cell, plays a pivotal role in ethanol production by this mutant. Conversion of glucose to two acetyl-CoA molecules by the glycolytic enzymes and PDH would yield four NADH molecules per glucose molecule, and these four NADH molecules can be oxidized using

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant genotype	Source or reference
E. coli K-12 strains		
W3110	Wild type	ATCC
AH241	W3110, Δ*ldhA*	19
AH242	W3110, Δ*ldhA* Δ(*focA-pflB*)-Km	19
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{W116}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	B. Wanner
JM109(λDE3)	F′ [*traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*)*M15*] Δ(*lac-proAB*) *glnV44 e14*$^-$ *gyrA96 recA1 relA1 endA1 thi hsdR17* (λDE3)	Promega
SE2377	AH242, *lpd102*(H322Y)	This study
SE2378	AH242, *lpd101*(E354K)	19
SE2382	AH242, *lpd101*(E354K)	This study
SE2383	AH242, *lpd102*(H322Y)	This study
SE2384	AH242, *lpd102*(H322Y)	This study
SE2385	AH242, *lpd101*(E354K)	This study
YK1	SE2378, Km^s	This study
YK29	AH242, Km^s	This study
YK87	YK1, Δ*adhE*-Km	This study
YK100	AH242, Δ*lpd*	This study
YK110	YK100, *lpd*$^+$	YK100 × P1(W3110)
YK111	YK100, *lpd101*	YK100 × P1(SE2378)
YK128	YK100, P*lpd*$^+$ (pKY32)	This study
YK129	YK100, P*lpd101* (pKY33)	This study
YK139	YK100, *lpd102*	YK100 × P1(SE2377)
YK141	YK100, *lpd101*	YK100 × P1(SE2382)
YK175	AH241, Δ*adhE*-Km	AH241 × P1(YK87)
YK176	YK141, Δ*adhE*-Km	YK141 × P1(YK87)
YK181	YK139, Δ*adhE*-Km	YK139 × P1(YK87)
Plasmids		
pKY10	pUC19, P*pdhR*$_{SE2378}$	This study
pKY13	pUC19, P*pdhR*$^+$	This study
pKY15	pTL61t, P*pdh*$_{SE2378}$-*lacZ*	This study
pKY17	pTL61t, P*pdh*$_{W3110}$-*lacZ*	This study
pKY32	pTrc99a, *lpd*$^+$ *bla*	This study
pKY33	pTrc99a, *lpd101 bla*	This study
pKY36	pET15b, *lpd*$^+$ *bla*	This study
pKY37	pET15b, *lpd101 bla*	This study
pKY38	pET15b, *lpd102 bla*	This study
Phages		
λYK1	λ(P*pdh*$_{W3110}$-*lacZ*)	This study
λYK2	λ(P*pdh*$_{SE2378}$-*lacZ*)	This study

the two acetyl-CoA molecules as the electron acceptors and alcohol dehydrogenase as the catalyst, with production of two equivalents of ethanol. For PDH to be active in an anaerobic cell, the LPD component of the PDH complex is expected to have lost at least part of its sensitivity to NADH inhibition.

Based on the DNA sequence, we localized the mutations in the ethanologenic *E. coli* mutants to a single change in the LPD amino acid sequence. The results presented in this paper show that the PDH from two such ethanologenic mutants, strains SE2377 and SE2378, are less sensitive to NADH inhibition. The alteration of the LPD and PDH complex to reduced sensitivity to NADH inhibition apparently allowed the enzyme to function in an anaerobic *E. coli* culture, which changed the fermentation profile of the mutant.

MATERIALS AND METHODS

Materials. Biochemicals were purchased from Sigma-Aldrich. Organic and inorganic chemicals were purchased from Fisher Scientific and were analytical grade. DNA restriction endonucleases, T4 DNA ligase, DNA polymerases, and other DNA modification enzymes and reagents were obtained from New England Biolabs Inc., Invitrogen, or Clontech Laboratories. The quantitative reverse transcription (RT)-PCR reagent was obtained from Bio-Rad Laboratories. Oligonucleotide primers were synthesized by Invitrogen or Sigma-Genosys.

Bacterial strains, bacteriophages, and plasmids. The bacterial strains, bacteriophages, and plasmids used in this study are listed in Table 1. All *E. coli* strains are derivatives of strain K-12.

Media and growth conditions. L broth (LB), used as the rich medium, and mineral salts medium were prepared as described previously (21). After the medium was autoclaved, sugars were added at final concentrations of 3 g/liter for aerobic growth and 10 g/liter for anaerobic growth. The media used for propagation of phages P1 and λ, as well as transduction, were prepared as described by Miller (25). Batch fermentation without pH control was carried out in screw-cap tubes (13 by 100 mm) filled to the top with the appropriate medium (19). The inoculum (1%, vol/vol) for the fermentations was grown aerobically for about 16 h. Antibiotics were added, as needed, at initial concentrations of 100 mg/liter of ampicillin and 50 mg/liter of kanamycin.

Genetic methods. Gene deletions in *E. coli* were constructed as described by Datsenko and Wanner (11). Appropriate genes were amplified by PCR and cloned into plasmid pCR2.1-TOPO (Invitrogen). After deletion of part of the gene, a DNA cassette containing a kanamycin resistance gene flanked by FRT sites was integrated into the deleted area. The antibiotic resistance gene with the flanking *E. coli* DNA was PCR amplified, and the PCR product was transformed into *E. coli* strain BW25113(pKD46) that was pregrown in LB containing arabinose as described previously (11). Transformants with the gene deletion were selected and verified by PCR. The deletion mutation was transduced by phage P1

to other genetic backgrounds before use. All molecular biology experiments were performed as described previously (28).

In vitro mutagenesis of *lpd*. The *lpd* gene in plasmid pKY32 was mutagenized by using either hydroxylamine or error-prone PCR. Hydroxylamine mutagenesis was performed as described by Davis et al. (12). Error-prone PCR was conducted as described previously (24) with the same primer set that was used for cloning the *lpd* gene into plasmid pET15b. In addition to the *Taq* polymerase buffer (New England Biolabs), the following ingredients were also added to increase the mutation rate: 0.8 mM dTTP, 0.8 mM dCTP, 4.8 mM $MgCl_2$, and 0.5 mM $MnCl_2$. PCR was performed using the following conditions with a Bio-Rad thermal cycler: 1 min at 95°C, followed by five cycles of 1 min at 95°C, 30 s at 45°C, and 2 min at 72°C, by 30 cycles of 1 min at 95°C, 30 s at 55°C, and 2 min at 72°C, and finally by 15 min at 72°C. The PCR product was purified, cloned into plasmid vector pTrc99a, and transformed into *E. coli* strain AH242. Transformants that grew anaerobically were selected, and the *lpd* gene in the plasmid was sequenced to identify the nature of the mutation.

Level of transcription of *pdh* operon. The *pdh* operon promoter DNA was removed from either plasmid pKY13 (W3110) or plasmid pKY10 (SE2378) after hydrolysis with BlpI and AflII. The DNA fragment was treated with the Klenow fragment of DNA polymerase and cloned into the SmaI site of plasmid pTL61t (23) upstream of a promoterless *lacZ* gene. The plasmid constructs (pKY15 for SE2378 P*pdh-lac* and pKY17 for W3110 P*pdh-lac*) were selected after transformation of *E. coli* TOP10 (Invitrogen) as blue colonies on LB containing ampicillin with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (40 μg/ml). The cloned *pdh* promoter DNA was sequenced to confirm the sequence. The P*pdh-lac* fusion was transferred to λRZ5 and transduced into *E. coli* as described previously (28).

Lysogens carrying the λP*pdh-lacZ* fusion were cultured under aerobic (LB) or anaerobic (LB containing glucose) conditions to the mid- to late exponential phase of growth. The β-galactosidase activity of the cells was determined as described by Miller (25). The specific activity of β-galactosidase was expressed in nmol · min^{-1} · mg cell $protein^{-1}$.

Quantitative RT-PCR. For isolation of total RNA, aerobic cultures were grown in 10 ml of LB in 250-ml flasks at 37°C with shaking at 200 rpm. Anaerobic cultures were grown in 9 ml of LB containing glucose in screw-cap tubes (13 by 100 mm) filled to the top. Cells were harvested at the early to mid-exponential phase of growth. Total RNA was extracted by the hot phenol method as described previously (34). Quantitative RT-PCR was performed as described previously (35).

LPD expression plasmids. Different alleles of the *lpd* gene were cloned into plasmid pTrc99a and expressed from a lactose-regulated promoter for complementation experiments. For construction of the plasmids, DNA encoding a specific *lpd* allele was amplified by PCR from appropriate *E. coli* genomic DNA. The forward primer (GCGACCATGGAGAAGGAGATATACCATGAGT ACT) contained an NcoI restriction site at the 5′ end (underlined), and the reverse primer (GCGAAAGCTTTTACTTCTTCTTCGCTTTCG) contained a HindIII restriction site at the 5′ end (underlined). A Shine-Dalgarno sequence (ribosomal binding site) was also located 7 nucleotides upstream of the start codon (ATG) in the forward primer. Both the PCR product and plasmid pTrc99A were hydrolyzed with restriction enzymes NcoI and HindIII and ligated to construct plasmids pKY32 and pKY33 containing lpd^+ and the *lpd101* allele, respectively.

For purification of LPD, the appropriate *lpd* allele was cloned into a phage T7-based expression vector. For construction of plasmids pKY36 (lpd^+), pKY37 (*lpd101*), and pKY38 (*lpd102*), the appropriate *lpd* gene was amplified by PCR with the following primers: forward primer GAGCCTCGAGATGAGTACTG AAATC and reverse primer GCGTGGATCCTTACTTCTTCTTCG. The forward primer contained an XhoI restriction site at the 5′ end (underlined), and the reverse primer had a BamHI restriction site at the 5′ end (underlined). The PCR products, digested with XhoI and BamHI, were ligated with plasmid pET-15b also digested with XhoI and BamHI. *E. coli* TOP10 cells were transformed with the ligation product, and the transformants were selected for resistance to ampicillin on LB containing ampicillin. The insert sequences in the plasmids were verified by sequencing the *lpd* gene.

Purification of LPD. For purification of LPD, the enzyme was produced in strain JM109(λDE3) transformed with plasmid pKY36, pKY37, or pKY38. A 500-ml culture in LB containing ampicillin in a 2.8-liter Fernbach flask was grown at 37°C with shaking at 250 rpm to an optical density at 420 nm of 0.6 (Beckman DU640 spectrophotometer). Arabinose (1.5%) was added to the culture to induce the T7 RNA polymerase (26). After 4 h of incubation at room temperature with shaking, cells were harvested by centrifugation (10,000 × *g*, 10 min, 4°C), washed twice with 25 ml of 50 mM potassium phosphate buffer (pH 8.0) (referred to as phosphate buffer below), and resuspended in 5 ml of the same buffer. All operations were conducted at 4°C. Cells were passed through a French pressure cell at 20,000 lb/in^2. The crude extract was clarified by centrifugation (30,000 × *g*, 45 min), and the supernatant was filtered through a 0.22-μm filter. The filtered protein solution was loaded onto a HiTrap chelating column (5 ml; General Electric) that was prewashed with 0.1 M $NiCl_2$ in the same buffer. Unadsorbed and loosely bound proteins were removed from the column by washing with 5 column volumes of phosphate buffer, followed by 5 column volumes of phosphate buffer with 50 mM imidazole. His-tagged LPD protein was eluted with a 50 mM to 0.5 M imidazole gradient in phosphate buffer. All the fractions containing LPD activity were combined. The N-terminal His tag was cleaved off the protein by incubation with thrombin (150 U; General Electric) at 4°C overnight. Thrombin and the small peptide were removed by gel filtration through a Sephacryl S-200 HR column (2.6 by 60 cm; General Electric) that was preequilibrated with phosphate buffer with 0.1 M NaCl. The protein was eluted with phosphate buffer containing 0.1 M NaCl. All the fractions with LPD activity were combined and dialyzed against phosphate buffer. The purity of the protein was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Both the native LPD protein and the mutated forms of LPD were purified by the same method. Since the purified LPD from strain SE2377, encoded by the *lpd102* allele, did not have detectable activity, purification of this LPD allele was followed by SDS-PAGE.

Purification of PDH complex. The PDH complex was purified as described by Bisswanger, with minor modifications (3), from strains YK175 (native protein), YK176 [*lpd101* allele; LPD(E354K)], and YK181 [*lpd102* allele; LPD(H322Y)]. Cells were cultured in 6 liters of glucose-mineral salts medium (1 liter per 2.8-liter Fernbach flask). When the culture reached an optical density at 420 nm of about 2.0 (Beckman DU640; late exponential phase of growth), cells were harvested by centrifugation (10,000 × *g*, 10 min, 4°C), washed with 100 ml of phosphate buffer, and resuspended in 20 ml of phosphate buffer. Cells were lysed by passage through a French pressure cell (20,000 lb/in^2) in the presence of a protease inhibitor cocktail (5 ml/20 g [wet weight] of cells; Sigma). DNase I and RNase A were each added to the extract in a centrifuge tube at a concentration of 100 μg/ml and incubated at 37°C for 1 h with gentle mixing to reduce the viscosity. All operations after this step were performed at 4°C. The cell extract was centrifuged at 12,000 × *g* for 30 min to remove cell debris. The supernatant was then centrifuged at 150,000 × *g* for 4 h to sediment the PDH complex. The supernatant was immediately decanted, and the pellet was dissolved in 6.0 ml of phosphate buffer for 2 h with gentle mixing on a rocker. The protein solution was centrifuged again at 12,000 × *g* for 15 min to remove particulates that did not dissolve. The supernatant was chromatographed through a hydroxyapatite column (1.5 by 12.0 cm; Bio-Rad) that was equilibrated with phosphate buffer. The protein was eluted from the column with a linear 50 to 500 mM phosphate gradient in phosphate buffer at pH 8.0. Fractions with PDH activity were combined, dialyzed against phosphate buffer, and concentrated. The concentrated protein solution was further purified with a gel filtration column (Sephacryl S-500HR; 2.6 by 35 cm) with phosphate buffer as the eluent. Fractions with activity were pooled and used immediately for the enzyme assay.

Enzyme activity. LPD activity was assayed as described previously (37). The standard reaction mixture (1.0 ml) for the forward reaction contained 0.1 M KH_2PO_4 (pH 8.0), 3 mM NAD^+, 3 mM DL-dihydrolipoic acid, 1.5 mM EDTA, and the appropriate amount of enzyme. One unit of enzyme activity was defined as the production of 1 μmol NADH · min^{-1} · mg $protein^{-1}$. The standard reverse reaction mixture (1.0 ml) contained 0.1 M KH_2PO_4 (pH 8.0), 0.1 mM NAD^+, 0.1 mM NADH, 3 mM DL-lipoamide, and 1.5 mM EDTA. Enzyme assays were performed at room temperature, and the rate of NADH oxidation was monitored over time. One unit of enzyme activity was defined as the oxidation of 1 μmol NADH · min^{-1} · mg $protein^{-1}$.

PDH was assayed both in crude extracts and using purified protein. A standard assay for determination of the activity of the PDH complex in crude extract was based on pyruvate-dependent reduction of NADH at 340 nm (ε = 6,220 M^{-1} cm^{-1}) at room temperature, as described by Hinman and Blass (17). Each 1-ml reaction mixture contained thiamine pyrophosphate (0.2 mM), CoA (0.1 mM), $MgCl_2 \cdot 6H_2O$ (1 mM), dithiothreitol (0.3 mM), NAD^+ (2.5 mM), bovine serum albumin (100 μg/ml), and crude extract or purified protein in 50 mM potassium phosphate buffer (pH 8.0). The reaction was started by addition of pyruvate (5 mM). Enzyme activity was expressed in μmol NADH produced · min^{-1} · mg $protein^{-1}$. The effect of NADH on enzyme activity was determined using the same reaction mixture with addition of various concentrations of NADH.

PDH activity in the crude extracts was also measured by using a partial reaction catalyzed by the pyruvate decarboxylase/dehydrogenase (E1 activity) in crude extracts (13). One milliliter of reaction mixture contained phosphate buffer (50 mM, pH 7.0), $MgCl_2 \cdot 6H_2O$ (12.5 mM), thiamine pyrophosphate (0.18 mM), CoA (0.175 mM), NAD^+ (2.0 mM), potassium ferricyanide (1.0 mM), pyruvate

TABLE 2. PDH mRNA, transcription, and protein levels in aerobically and anaerobically grown *E. coli* wild-type strain W3110 and ethanologenic mutant strain SE2378

Strain	Relative mRNA level[a]		β-Galactosidase activity[b]		PDH activity[c]	
	With O_2	Without O_2	With O_2	Without O_2	With O_2	Without O_2
W3110	1.00	0.98	600	630	370	185
SE2378	0.71	0.77	570	680	240	200

[a] Relative mRNA levels were determined by quantitative RT-PCR. The level of *aceE* mRNA in wild-type strain W3110 grown under aerobic conditions was defined as 1.0, and the relative levels of *aceE* mRNA for the other growth conditions and strain SE2378 were determined.

[b] β-Galactosidase activities of P*pdh-lacZ* fusions using λYK1 and λYK2 at λ*att* are expressed in nmol · min^{-1} · mg $protein^{-1}$.

[c] The PDH activity is the PDH (E1) activity of the PDH complex and is expressed in nmol ferricyanide reduced · min^{-1} · mg $protein^{-1}$. See Materials and Methods for other details.

(5.0 mM), and crude extract. The reaction was initiated by addition of pyruvate. The rate of reduction of ferricyanide was monitored over time at 430 nm (ε = 1,030 M^{-1} cm^{-1}). One unit of enzyme activity was defined as the reduction of 1 μmol ferricyanide · min^{-1} · mg $protein^{-1}$.

The reported results are data from a typical experiment that was repeated at least three times, and the variation in the experimental results was less than 10%. Kinetic properties of both LPD and PDH were determined as described by Cornish-Bowden (10) using the initial linear rates of the reactions.

Analytical methods. The NAD^+ concentration was determined using an Enzychrom NAD^+/NADH assay kit (Bioassay Systems, Hayward, CA). Sugars and fermentation products were analyzed by high-performance liquid chromatography as described previously (19). The nucleotide sequence of DNA was determined by the Interdisciplinary Center for Biotechnology Research DNA sequencing core facility at the University of Florida. Protein concentrations were determined using Coomassie blue G-250 as described by Bradford (5) with bovine serum albumin as the standard. SDS-PAGE was performed with 12.5% polyacrylamide gels as described by Laemmli (20).

RESULTS AND DISCUSSION

An *E. coli* mutant (strain AH242) that lacks pyruvate-formate lyase (*pflB*) and fermentative lactate dehydrogenase (*ldhA*) activities is defective for anaerobic growth (9, 19). We derived a set of mutants (e.g., strain SE2378) of strain AH242 that grew anaerobically and produced ethanol as the fermentation product (19). The mutation in strain SE2378 was mapped in or near the genes that encode the components of the PDH complex (*pdh* operon; *pdhR*, *aceE*, *aceF*, and *lpd*). Since the expected [NADH]/[NAD^+] ratio in anaerobic cells is higher than that in aerobic *E. coli* cells (13), operation of this new ethanol production pathway in strain SE2378 that includes glycolysis, PDH, and alcohol dehydrogenase suggests that the activity of the PDH complex is less sensitive or insensitive to inhibition by NADH, in addition to its optimal expression.

Expression of PDH in an anaerobic culture of *E. coli*. The results presented in Table 2 show that the relative mRNA level of the *aceE* gene (the second gene in the *pdh* operon) encoding the E1 enzyme of the PDH complex is independent of the presence of O_2 during growth of *E. coli* strain W3110. Since the *pdhR*, *aceE*, *aceF*, and *lpd* genes are transcribed as one transcript (27), it is apparent that all the genes encoding the PDH complex are transcribed under both aerobic and anaerobic growth conditions. Although the *aceE* mRNA level of strain SE2378 was also not altered by the level of O_2 during growth, this mRNA level was slightly lower than that of the wild-type strain W3110. The fact that this mRNA is further translated in cells grown under aerobic and anaerobic conditions is shown by the P*dhR-lac* fusion-based production of β-galactosidase activity and the presence of PDH activity in extracts (Table 2). In wild-type strain W3110, the level of PDH activity of anaerobic cells was about 50% of the level of activity of aerobic cells, while the levels of transcription in the two growth conditions were about the same. It is possible that the NADH-inhibited protein was subject to proteolysis in the anaerobic cells. Although the level of PDH activity in strain SE2378 grown under aerobic conditions was only about 65% of the level of activity in aerobically cultured strain W3110, this level of activity was not that dissimilar from the level in either strain grown anaerobically. These results show that both the wild-type and mutant *E. coli* strains produced PDH during anaerobic growth. The inability of strain AH242 (an *ldhA pflB* derivative of strain W3110) to grow under anaerobic conditions suggests that the PDH activity is negligible during anaerobic growth of strain W3110 due to inhibition of the enzyme complex by NADH. Similar conclusions were also reached by Snoep et al. based on the fermentation profile of *E. coli* B (33).

PDH complex is less sensitive to NADH inhibition in strain SE2378. For the PDH to be active under anaerobic growth conditions, the mutated form of the enzyme in strain SE2378 needs to be significantly less sensitive to NADH inhibition than the native enzyme. When the PDH activity of strain W3110 was assayed in crude extracts, the enzyme was completely inhibited by NADH at a concentration of about 80 μM, corresponding to an [NADH]/[NAD^+] ratio of about 0.04. At this ratio of [NADH] to [NAD^+], the PDH complex in the crude extract of strain SE2378 was fully active (Fig. 1). Increasing the NADH concentration to obtain a higher ratio did inhibit the enzyme from the mutant, indicating that the mutation did not completely mitigate the NADH inhibition. Taken together, the results presented in Table 2 and Fig. 1 show that the observed anaerobic growth phenotype of strain SE2378 is apparently due to the mutational alteration of the PDH activity.

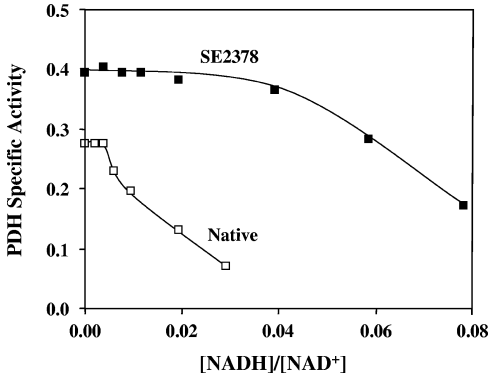

FIG. 1. NADH sensitivity of the PDH complex from *E. coli* wild-type strain W3110 (Native) and an ethanologenic mutant, strain SE2378. Crude extracts were used to assay for PDH complex activity (NADH production) as described in Materials and Methods. The NAD^+ concentration in the assay mixture (2 mM for the native enzyme and 1 mM for the enzyme from strain SE2378) was at least five times the K_m value for each enzyme. The NADH concentration was varied to obtain the indicated ratios. Specific activity is expressed in μmol min^{-1} mg $protein^{-1}$.

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W3110 (wt)    321 AHKGVHEGHV AAEVIAGKKH YFDPKVIPSI AYTEPEVAWV
SE2377        321 .Y........ .......... .......... ..........
SE2378        321 .......... .......... .......... ...K......
SE2382        321 .......... .......... .......... ...K......
SE2383        321 .Y........ .......... .......... ..........
SE2384        321 .Y........ .......... .......... ..........
SE2385        321 .......... .......... .......... ...K......
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FIG. 2. Amino acid sequence alignment of LPD from *E. coli* wild-type (wt) strain W3110 and various ethanologenic mutants. A period indicates that the amino acid is same as the amino acid in the wild type. All the other amino acids that are not listed are the same in the LPD from the six mutants and strain W3110.

Identification of the mutation in *lpd*. To localize the mutation that contributed to the reduced sensitivity of PDH to NADH inhibition, the genes comprising the entire *pdh* operon from six independently isolated ethanologenic *E. coli* mutants were sequenced. Based on the DNA sequence, a single mutation in *lpd* was identified in each of the six mutants (Fig. 2). Three mutants, strains SE2377, SE2383, and SE2384, had a base change from C to T at position 964 (A in the ATG codon of the LPD gene was defined as position 1; *lpd102*), resulting in a change of the histidine at position 322 to tyrosine (Fig. 2). The other three mutants, strains SE2378, SE2382, and SE2385, had a different mutation (G to A at position 1,060; *lpd101*), resulting in a change of the glutamate at position 354 to lysine (Fig. 2). Only one of the six mutants, strain SE2378, also carried two mutations in the *pdhR* gene (base change T34C leading to amino acid change S12P; insertion of a TGC sequence between positions 352 and 353, resulting in insertion of leucine between amino acids 117 and 118 in the native protein) besides the E354K change in the LPD protein. In addition, a G-to-A base change upstream of the *aceE* gene at position −59 from the translational start (A of ATG) was also observed in strain SE2378. These secondary mutations in strain SE2378 may have altered the level of expression of the PDH complex in the cell since the PdhR protein is a known regulator of the *pdh* operon (16). The mutations in LPD are in agreement with the observed lower sensitivity of the PDH complex in cell extracts to NADH inhibition since the LPD enzyme is the known site of NADH inhibition (29, 30, 38).

To confirm that the observed phenotype of anaerobic growth is due only to the *lpd* mutation and is not associated with a second mutation in an unlinked gene, the following genetic experiment was performed. In the first step, the *lpd* gene of the *ldhA pflB* double mutant strain AH242, the parent strain used in construction of the ethanologenic derivatives, was deleted (strain YK100). Strain YK100 is defective for anaerobic growth due to the *ldhA* and *pflB* mutations and defective for aerobic growth in glucose-mineral salts medium due to the absence of PDH activity (*lpd* deletion). Wild-type and *lpd101* alleles were either transduced into strain YK100 or introduced via plasmids, and the derivatives with LPD activity were selected for growth under aerobic conditions in glucose-mineral salts medium (Table 3). Only the transductants carrying the *lpd101* allele from either strain SE2378 or SE2382 grew anaerobically, while the transductants carrying the wild-type lpd^+ gene did not grow anaerobically (Table 3). Strain SE2382 is distinguished from strain SE2378 by its lack of the additional mutations found in the *pdh* operon of strain SE2378. These results show that the mutation in *lpd* is responsible for the observed phenotype. Similar results were also obtained when the Δ*lpd* mutation in strain YK100 was complemented with two alleles, lpd^+ and *lpd101* from a plasmid, expressed from a *trc* promoter with isopropyl-β-D-thiogalactopyranoside (IPTG). The complementation experiments confirmed that the mutation in the *lpd* gene alone supports anaerobic growth of an *ldhA pflB* double mutant. As expected, transductants of YK100 with the *lpd102* allele (YK139) also grew anaerobically (data not shown), confirming that the two *lpd* mutations (*lpd101* and *lpd102*) are interchangeable.

In order to isolate additional LPD mutations that also would support anaerobic growth of strain AH242 but may map at different parts of LPD, the *lpd* DNA was specifically mutagenized with hydroxylamine or by error-prone PCR. The PCR mutagenesis did not yield any *lpd* mutation that supported anaerobic growth of strain AH242 (from a total of about 4,000 transformants). Hydroxylamine treatment of *lpd* DNA did yield five additional mutants, and the mutation in all five was the same E354K mutation found in strain SE2378.

LPD activity of strain SE2378 is insensitive to NADH inhibition. Since, the *lpd* mutation in all six ethanologenic strains supported anaerobic growth in the *ldhA pflB* genetic background, the LPD protein was purified from the wild type (native enzyme) and a representative strain SE2378 (mutated enzyme; E354K), and the kinetic properties were determined (Fig. 3 and 4).

The native enzyme presented typical Michelis-Menton-type kinetics with respect to NAD^+. However, addition of NADH to the reaction mixture progressively inhibited the enzyme

TABLE 3. Anaerobic growth and fermentation profiles of *E. coli* strains with different *lpd* alleles[a]

Strain	*lpd* allele	Anaerobic growth	Concn of fermentation products (mM)				
			Ethanol	Acetate	Formate	Succinate	Lactate
W3110	lpd^+	+	7.3	9.3	2.6	4.2	18.5
SE2378	*lpd101*	+	107.3	4.7	0.0	4.1	0.8
YK110	lpd^+	−	NG	NG	NG	NG	NG
YK111	*lpd101*[b]	+	125.8	2.2	0.0	5.0	0.3
YK141	*lpd101*[b]	+	114.5	3.4	0.0	6.0	0.3
YK128	Plpd^+	−	NG	NG	NG	NG	NG
YK129	P*lpd101*	+	68.0	3.6	0.0	11.9	0.8

[a] See Table 1 for a description of strain construction. Anaerobic growth and fermentation were determined using LB containing glucose (1%) in batch fermentations without pH control. Glucose was not completely fermented by strain W3110 due to the accumulation of organic acids that lowered the culture pH to less than 5.0. See text for other details. NG, no growth.

[b] The *lpd101* allele in strains YK111 and YK141 was transduced from strains SE2378 and SE2382, respectively.

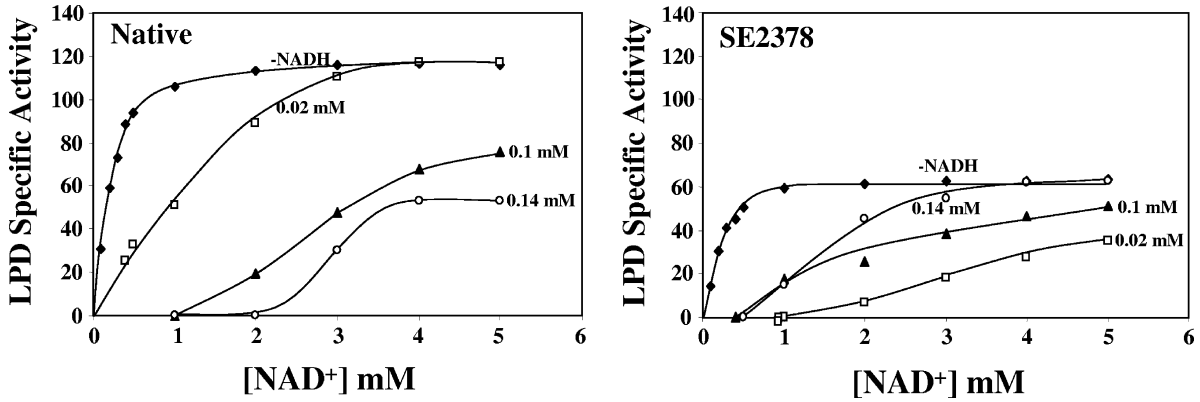

FIG. 3. NADH inhibition of LPD from *E. coli* wild-type strain W3110 (Native) and mutant strain SE2378. The rate of the forward reaction of LPD as a function of [NAD^+] was determined without NADH and with the indicated concentrations of NADH. Specific activity is expressed in μmol min^{-1} mg $protein^{-1}$.

activity (Fig. 3). The specific activity of the mutated form of the enzyme was only about 50% of that of the native enzyme, and this altered enzyme was surprisingly more sensitive to inhibition at a lower NADH concentration (0.02 mM) than at higher concentrations of NADH. At an NAD^+ concentration of 2 mM, 0.14 mM NADH completely inhibited the activity of the native enzyme, while at the same concentration of NAD^+, 0.14 mM NADH inhibited the mutated form of the enzyme only by about 25%. At 4 mM NAD^+, the mutated LPD was not inhibited by 0.14 mM NADH, while the native enzyme activity was only about 40% of the control activity without NADH. The K_m for NAD^+ (0.4 mM) was the same for both forms of the enzyme and is within the range of the reported K_m values for the *E. coli* LPD that varied as a function of pH and NADH concentration (0.09 to 1.0 mM) (29). The K_i for NADH for the native enzyme was 5.2 μM, and this value is close to the value previously reported, 9 μM (30). Due to the complexity of NADH inhibition of the mutated LPD activity (Fig. 3), K_i was not determined for the LPD(E354K) enzyme.

The reverse reaction of native LPD, lipoamide + NADH $\rightarrow$ dihydrolipoamide + NAD^+, depends on NAD^+ as an activator (38). Reversal of NADH inhibition of the enzyme activity by NAD^+ was biphasic. The first phase showed a gradual increase in activity to about 25 U when the added NAD^+ concentration matched that of the substrate, NADH. The activity of the second phase of NAD^+-dependent activation of the LPD reverse reaction was significantly higher, reaching the maximum value, about 70 U, when the NAD^+ concentration was 1.5 times the concentration of NADH (Fig. 4). The E354K form of the enzyme had about 55 U of activity in the absence of added NAD^+, which was about 75% of the maximum activity (about 75 U), which was reached with addition of a very small amount of NAD^+ (20 μM). Complete activation of the native enzyme required at least 150 μM NAD^+, an amount that was 7.5-fold larger than the amount needed for activation of the E354K form of the enzyme. These results further demonstrate that the sensitivity of LPD to inhibition by NADH is minimal in strain SE2378. An alternative possibility, the possibility that the E354K form of LPD carries a tightly bound NAD^+ that overcomes the need for external addition of NAD^+ to reverse NADH inhibition, can be ruled out due to the absence of detectable NAD^+ in the purified protein (data not shown).

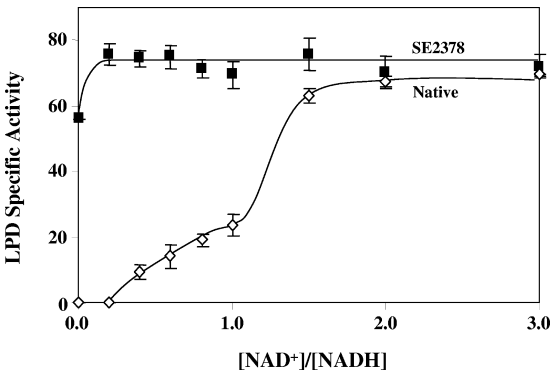

FIG. 4. Insensitivity of the LPD (reverse reaction) from mutant strain SE2378 to NADH. The reverse reaction of LPD was determined with 0.1 mM NADH and different concentrations of NAD^+ to obtain the indicated ratios. Specific activity is expressed in μmol min^{-1} mg $protein^{-1}$. Native, wild-type strain.

H322Y form of LPD is inactive upon purification. Three of the mutants isolated as derivatives of strain AH242 that could grow anaerobically carried a mutation that changed the amino acid at position 322 from histidine to tyrosine (H322Y; *lpd102*) (Fig. 2). The *lpd102* DNA was cloned into an expression vector, and the H322Y form of the LPD protein was expressed in recombinant *E. coli*. Although the cell extract had LPD activity, upon purification the protein lost the activity and associated flavin adenine dinucleotide (FAD) at the gel filtration step. Attempts to reconstitute the enzyme with added FAD as described by Lindsay et al. (22) failed to yield a protein with activity. This form of the enzyme was not studied further.

***E. coli* PDH complex with LPD mutation is less sensitive to NADH inhibition.** In order to confirm that the lower sensitivity of the LPD from strain SE2378 to NADH inhibition is translated to the entire PDH complex, the PDH complex was purified from the wild type and two mutants, strains SE2377 and SE2378, representing the two alleles. Kinetic properties of the PDH from these strains are presented in Table 4. The native form and the two mutated forms of the enzyme had similar

TABLE 4. Kinetic characteristics of the PDH complex isolated from *E. coli* wild-type strain W3110 and strains SE2377 (H322Y) and SE2378 (E354K) with a mutation in LPD[a]

PDH complex	K_m (NAD^+) (mM)	K_m (pyruvate) (mM)	K_i (NADH) (μM)	V_{max} (μmol · min^{-1} · mg $protein^{-1}$)
Native	0.07	0.40	1.00	46.64
H322Y	0.11	0.48	9.60	26.70
E354K	0.08	0.30	10.00	30.28

[a] Enzymes were purified from *E. coli* strains YK175 (native protein), YK181 [LPD(H322Y)], and YK176 [LPD(E354K)]. See Table 1 for the genotypes of the strains.

affinities for the substrates, NAD^+ and pyruvate. The K_m values for pyruvate and NAD^+ are within the range of K_m values reported for the *E. coli* PDH complex (0.2 to 0.4 mM for pyruvate and 10 μM for NAD^+) (4, 36). However, the native enzyme had a higher V_{max} than the two mutated forms of the enzyme. Apparently, the lower specific activity of the LPD than of the native enzyme (Fig. 3) is translated to lower PDH activity. It is interesting that although the LPD with the H322Y mutation (strain SE2377) lost FAD upon purification, the PDH complex with the same mutated form of the LPD was active after purification.

The two mutated forms of the PDH complex had about 10-fold-higher K_i values for NADH, in agreement with the observation that the PDH complex from the two mutants is less sensitive to inhibition by NADH (Table 4 and Fig. 5). The native PDH was completely inhibited at an NADH concentration of 0.06 mM, while the two mutated forms of PDH retained about 70 to 85% of the activity at the same NADH concentration. Even at a higher NADH concentration, the two mutated forms of PDH retained about 70% of the enzyme activity.

The intracellular [NADH]/[NAD^+] ratio of *E. coli* grown under aerobic conditions was reported to be about 0.03 to 0.05 (8, 13, 33). At this ratio, the PDH complex retained almost 90% of the maximal activity. The [NADH]/[NAD^+] ratio of an anaerobic cell is expected to be higher than that of an aerobic cell (0.7 to 0.8) (13, 33). Even at the lower reported ratio of 0.3, the PDH complex is not active in vitro (Fig. 5), supporting the inactive nature of the PDH complex in anaerobic *E. coli*. Although it is difficult to extrapolate from in vitro enzyme activity to in vivo levels, it should be noted that the PDH complex, although present, failed to support anaerobic growth of strain AH242 that lacked lactate dehydrogenase and pyruvate-formate lyase (19). The specific mutation, either H322Y or E354K, that made the LPD less sensitive to NADH inhibition also reduced the NADH sensitivity of the PDH complex and allowed the enzyme to support anaerobic growth of *E. coli* strain SE2378.

Histidine at position 322 was conserved in all LPD sequences tested, including the human LPD sequence, indicating the importance of this amino acid in the activity of LPD and PDH. Although the glutamate at position 354 was conserved in the LPD of all tested bacteria in the family *Enterobacteriaceae*, it is not conserved across bacterial families. However, a significant level of sequence identity can be found in this region; proline at position 348 is conserved in all the LPD sequences tested, including the human sequence. A glutamate is located at position 356, with few exceptions. The specific role that these amino acids play in the structure or catalysis of LPD is unclear, but these amino acids apparently are important for the normal function of the enzyme.

Although several spontaneous and induced mutations that affect the activity of LPD were reported in several organisms, none of these mutations was at the two positions reported in this study (1, 2, 6, 7, 18). In addition, none of the reported LPD mutations were reported to significantly alter the NADH sensitivity of the PDH complex. The two LPD mutations described in this study are unique in this regard in that they affect the NADH sensitivity of the protein with minimal effects on the overall activity of the protein. The inhibition of PDH (LPD) by NADH is probably caused by overreduction of the redox centers in LPD by NADH, making the enzyme inactive (14). NADH also increased the K_m for NAD^+, and there was an associated decrease in the LPD activity (29). It is possible that the two mutated forms of LPD described in this study have a higher dissociation constant (K_d) for NADH and/or a reduced rate of FAD reduction by NADH compared to the native LPD. The mechanism of this reduction in NADH sensitivity has not been determined yet.

Conclusion. The results presented here show that a mutation in LPD led to a PDH complex that is less sensitive to inhibition by NADH. The altered enzyme was active even in

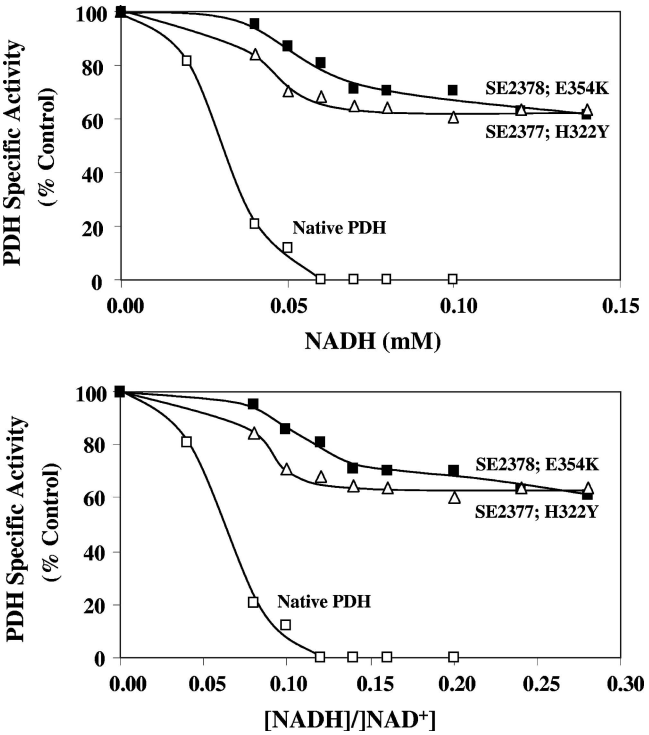

FIG. 5. Differential inhibition of the PDH complex from wild-type *E. coli* strain W3110 (Native) and the two LPD mutant derivatives by NADH. The NAD^+ concentration (0.5 mM) was at least five times the K_m value. The specific activity of the native enzyme complex without NADH was 40.16 U. The specific activities of the two PDH complexes with LPD mutations H322Y and E354K without NADH were 17.74 and 31.00 U, respectively.

the presence of a higher level of NADH in the cell and supported anaerobic growth of strains SE2378 and SE2382 and other similar mutants. The combined functions of glycolysis and the PDH complex in these mutants produced four NADH molecules during conversion of one glucose molecule to two acetyl-CoA molecules. These four NADH molecules were reoxidized by reduction of the two acetyl-CoA molecules to two ethanol molecules by the alcohol/acetaldehyde dehydrogenase (*adhE*), the observed phenotype of strain SE2378 and other mutants. These results raise an interesting possibility, that a similar alteration in the LPD of other organisms could also lead to PDH complexes that are less sensitive to NADH inhibition and active during anaerobic growth. The presence and functional activity of such an NADH-insensitive PDH may have significant unexplored physiological and biotechnological applications.

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