Metabolic Flux Control at the Pyruvate Node in an Anaerobic 

*Escherichia coli* with Active Pyruvate Dehydrogenase

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Running Title: Carbon flow at the pyruvate node in anaerobic *E. coli* with PDH activity

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Abstract

During anaerobic growth of *Escherichia coli*, pyruvate formate-lyase (PFL) and lactate dehydrogenase (LDH) channel pyruvate towards a mixture of fermentation products. We have introduced a third branch at the pyruvate node in a mutant of *E. coli* with a mutation in pyruvate dehydrogenase (PDH*) that renders the enzyme less sensitive to inhibition by NADH. The key starting enzymes of the three branches at the pyruvate node in such a mutant, PDH*, PFL and LDH, have different metabolic potential and kinetic properties. In such a mutant (strain QZ2), pyruvate flux through LDH was about 30% with the remainder through PFL indicating that LDH is a preferred route of pyruvate conversion over PDH*. In a *pfl* mutant (strain YK167) with both PDH* and LDH activities, flux through PDH* was about 33% of the total confirming the ability of LDH to outcompete the PDH pathway for pyruvate in vivo. Only in the absence of LDH (strain QZ3), pyruvate carbon was equally distributed between PDH* and PFL pathways. A *pfl* mutant with LDH and PDH* activities as well as a *pfl*, *ldh* double mutant with PDH* activity had a surprisingly low $Y_{\text{ATP}}$ (about 7.0 grams cells per mole ATP) compared to 10.9 grams cells per mole ATP for the wild type. The lower $Y_{\text{ATP}}$ suggests the operation of a futile energy cycle in the absence of PFL in this strain. An understanding of the controls at the pyruvate node during anaerobic growth is expected to provide unique insights into rational metabolic engineering of *E. coli* and related bacteria for production of various bio-based products at high rate and yield.
Introduction

In *Escherichia coli* as well as in other aerobic organisms, sugars such as glucose are metabolized in two separate steps; glycolysis that converts glucose to pyruvate and TCA cycle enzymes that oxidize acetyl-CoA to CO$_2$ (5, 9). The enzyme pyruvate dehydrogenase complex (PDH) connects the glycolytic reactions to TCA cycle enzymes by catalyzing the production of acetyl-CoA from pyruvate. Because of its unique central role in metabolism, PDH is regulated both at the genetic and biochemical level (7, 12, 27, 33, 34). The NADH generated during the complete oxidation of sugar is reoxidized to NAD$^+$ by O$_2$ through the respiratory electron transport pathway with accompanying energy production (11). Optimum coupling of these enzyme reactions helps to maintain the internal ratio of [NADH]/[NAD$^+$] (redox balance) and [ATP]/([ADP]+[AMP]) to support growth at the highest rate.

Absence of O$_2$ or other external electron acceptor during growth of *E. coli* (anaerobic conditions) forces the bacterium to minimize the contribution of the TCA cycle enzymes to that of biosynthesis from that of catabolism (4, 14). Under these conditions, pyruvate or acetyl-CoA derived from pyruvate serves as the electron acceptor (reduced to lactate and ethanol, respectively) to maintain the redox balance. The enzymes responsible for redox balance in anaerobic *E. coli* are pyruvate formate-lyase (PFL), lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH-E). The main products of fermentation of *E. coli* are a mixture of organic acids such as acetate, lactate and formate, in addition to ethanol (2, 4). Succinate, derived from phosphoenolpyruvate, is a minor product of fermentation and normally accounts for less than 5% of the total products produced from glucose by the culture.
Anaerobic growth of *E. coli*, compared to aerobic growth, is also limited by energy leading to an increase in glycolytic flux (19). Conversion of pyruvate to acetate and ethanol yields an additional ATP per glucose suggesting that this would be the preferred route for pyruvate oxidation during anaerobic growth. This is accomplished by the pyruvate formate-lyase (PFL) dependent production of acetyl-CoA and further conversion to acetate (Fig. 1). This preference for PFL has been demonstrated with several bacteria under carbon limitation condition either imposed in a chemostat or in the presence of a poor carbon source (10, 20, 23). This additional ATP also elevates the ATP yield per glucose to 3 with an increase in growth rate and has been shown to be essential for anaerobic growth of *E. coli* in xylose-mineral salts medium (13). Absence of this third ATP in a *pfl* mutant was reported to increase glycolytic flux to lactate to compensate for this decrease in ATP yield per glucose (39). However, flow of pyruvate carbon to acetate is tempered by the need to maintain redox balance and this is achieved by the conversion of a second acetyl-CoA to ethanol by ADH-E. Under conditions of energy excess due to declining growth rate, lactate production is expected to support redox balance maintenance without the additional ATP from the PFL-ADH-E pathway (Fig. 1). Production of this mixture of products in appropriate ratio helps to maintain the redox balance under anaerobic condition while also maximizing the ATP yield per glucose to support high growth rate and cell yield.

A PDH-based fermentation reaction to ethanol that can also help maintain cellular redox balance in an anaerobic cell has not evolved in *E. coli* or other closely related bacteria. The PDH activity is inhibited by NADH, normally found to be higher in anaerobically growing culture compared to an aerobic culture (12, 18, 34, 35). Based on genome sequences available in
Genbank, the genes encoding the components of PDH are not found in strict anaerobic bacteria.

We have recently described a mutation (*lpd101*) in the dihydrolipoamide dehydrogenase (LPD) of the PDH that allowed the enzyme to function in the anaerobic cell (designated here as PDH*) (17, 18). With this altered PDH* complex, an anaerobic cell can have three different pathways for pyruvate metabolism (Fig. 1). The three main enzymes that utilize pyruvate as substrate, PDH*, PFL and LDH, have different apparent $K_m$ values for pyruvate (0.4, 2.0 and 7.2 mM, respectively) (1, 18, 37, 41). PDH requires NAD$^+$ for activity (apparent $K_m$, 0.07 mM) while LDH is dependent on NADH (apparent $K_m$, 0.2 mM) as the second substrate (18, 37).

The PDH* serves as the first enzyme in a pathway that oxidatively decarboxylates pyruvate to acetyl-CoA and NADH followed by reduction of the acetyl-CoA by alcohol dehydrogenase to ethanol in a two step process using 2 NADHs (Fig. 1). The NADH produced during conversion of $\frac{1}{2}$ glucose to acetyl-CoA dictates that the acetyl-CoA generated by PDH is used for redox balance (ethanol) and not for ATP generation (acetate), unless some of the NADH is used for biosynthesis by the growing cell (17). PDH* and LDH serve essentially the same physiological role in the cell, oxidizing NADH to support continued operation of glycolysis although it is not readily apparent with PDH*. This potential competition between the two enzymes has been eliminated in the wild type by inhibiting the activity of PDH by NADH (12, 18, 32). However, the in vivo role of PDH* in a mutant that has all three pathways has not been investigated since the flow of pyruvate through any of the three reactions during growth and post-growth fermentation of sugars to products is expected to be dependent on the redox state, ATP requirement and other physiological conditions of the anaerobic cell. Using a combination of
metabolic flux analysis and mutations in one or more of the genes encoding these enzymes, we have evaluated the flow of pyruvate carbon among the three potential pathways and these results are presented in this communication.
MATERIAL AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. All strains are derived from *E. coli* K-12 strain W3110 (ATCC 27325). Strain YK1 is a kanamycin sensitive derivative of strain SE2378 that carries the *lpd101* mutation described previously (17). Gene deletions were constructed using the method described by Datsenko and Wanner (6). Mutations were transduced using bacteriophage P1 as described previously (29).

Growth medium. L broth (29) supplemented with 20 g/L of glucose was used for all chemostat cultivations. Glucose concentration was increased to 50 g/L in batch fermentation experiments.

Bioreactor conditions. For metabolic flux analysis, fermentations were conducted under anaerobic chemostat conditions in a 2.5 L New Brunswick fermenter (Bioflo III; New Brunswick, NJ) with 1.2 L culture volume. Anaerobic conditions were established by sparging the culture with N$_2$ at a flow rate of 0.2 liters min$^{-1}$. The pH, temperature and agitation were maintained at 7.0, 37°C, and 250 RPM, respectively. Culture pH was maintained by addition of KOH. A dilution rate of 0.1 h$^{-1}$ was maintained throughout the experiment by controlling the medium feed rate. The continuous culture reached steady state after 5-6 residence times. Samples were taken every 5 hours for analysis. Three samples were taken from the steady state culture and the average of the three was used for the calculation. Deviation from this mean was less than 5% and the results from one representative complete experiment are presented. For batch fermentations, cells were cultivated with 50 g/L glucose starting with a 16 h old aerobic culture as the inoculum(1% v/v) as previously described (17). All fermentation experiments were repeated at least three times and the variation among experimental results was less than 15%. Results from
one representative experiment are presented in this communication.

**Enzyme activity.** A 250 ml culture from a batch fermentation in LB with glucose (30 g/L) was harvested at mid-exponential phase of growth and the cells were collected after centrifugation (10,000 x g, 20 min, 4°C). Cells were washed twice with 25 ml of 50 mM potassium phosphate buffer (pH 7.5) 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 psi. The crude extract was centrifuged first at 10,000 x g for 30 min and the supernatant was further clarified by centrifugation at 30,000 x g for 60 min. The supernatant was used for enzyme assays.

PDH activity was determined by monitoring pyruvate-dependent reduction of NAD\(^+\) at 340 nm (\(\varepsilon = 6,220 \text{ M}^{-1} \text{ cm}^{-1}\)) at room temperature, as described previously (18). LDH activity was determined by measuring pyruvate-linked NADH oxidation at 340 nm (43). ADH-E activity was assayed by measuring the increase in absorbance at 340 nm resulting from reduction of NAD\(^+\) in the presence of ethanol (http://www.worthington-biochem.com/ADH/assay.html). One unit of enzyme activity is one \(\mu\)mole of product produced min\(^{-1}\).

Total RNA was extracted from the same cultures used for enzyme activity determination and specific mRNA level in the RNA sample was determined as described by Kim et al. (18).

**Analytical methods.** Sugar and fermentation products were determined by high-performance liquid chromatography, using HP1090 (Agilent Technologies, Santa Clara, CA) fitted with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and UV (210 nm) and RI detectors, in series (38). Cell density was monitored at 420 nm (Beckman DU640; Fullerton, CA) and reported as cell dry weight (CDW). Cell dry weight of a culture at one OD at 420 nm equals...
0.22 g/L. Protein concentration was determined by Bradford method with bovine serum albumin as standard (3).

Metabolic flux analysis. Metabolic flux analysis was used for the calculation of in vivo fluxes (36). The methodology relies on the metabolite balances, biochemical constraints, and pseudosteady-state assumption for intracellular metabolites (40). The stoichiometric reactions presented in Fig. 2 were used for calculation of flux distributions based on experimental data. The resulting metabolic network consists of 13 reactions and 8 of them (J1, J6, J7, J8, J10, J11, J12 and J13) can be measured. All the lactate produced by the ldhA-plus strains was assumed to be produced by LDH (reaction J7). In the ldhA mutant, the lactate was assumed to be from the methylglyoxal pathway (reaction J13). In order to distinguish between the contribution of PDH* and PFL, further metabolism of formate was eliminated by introducing hypF mutation to remove formate hydrogen-lyase activity. In such a hypF mutant, the level of formate in the medium is a measure of PFL activity.

Materials. Inorganic salts, organic chemicals and medium components were purchased from either Fisher Scientific (Pittsburgh, PA) or Sigma Chemicals Co. (St. Louis, MO).
RESULTS AND DISCUSSION

Preferred pathway at the pyruvate node. In strain QZ2 that carries the lpd101 mutation rendering the PDH* functional during anaerobic growth, pyruvate flux through PDH* in batch cultures was minimal during anaerobic growth (Fig. 3A). Strain QZ2 produced acetate, ethanol, formate and lactate in pH-controlled batch fermentation. The specific rate of production of acetate, ethanol and formate during exponential phase of growth was 29.1, 27.2 and 51.5 mmoles h\(^{-1}\) (g CDW\(^{-1}\))\(^{-1}\), respectively. Presence of equimolar amounts of acetate and ethanol in the medium suggests that PFL-produced acetyl-CoA is the precursor for these two products. This is also confirmed by the specific rate of productivity of formate that is the combined rates of acetate and ethanol production, a measure of the PFL pathway. The specific rate of lactate production (26.1 mmoles h\(^{-1}\) (g CDW\(^{-1}\))\(^{-1}\)) during this growth phase was about 50% of the formate production rate. Presence of lactate in the fermentation broth suggests that a significant fraction of reductant generated during glycolysis was channeled to reduce pyruvate to lactate to maintain redox balance. An interesting observation is the absence of PDH*-based acetyl-CoA production in this culture since this acetyl-CoA can be converted to ethanol while also supporting optimum redox balance (17, 18). Although PDH* requires NAD\(^{+}\) for activity in contrast to LDH, a coupled PDH* and ADH pathway (pyruvate to ethanol) does oxidize one net NADH, as does LDH (Fig. 1). However, in vivo, LDH appears to be the preferred enzyme for NADH oxidation in the cell. In support of this pyruvate flux distribution, the PDH activity of strain QZ2 (0.14 unit mg protein\(^{-1}\)) was found to be only about 30% of an isogenic “wild type” strain PMD23 (0.46 unit mg protein\(^{-1}\)) (Table 2).
Pyruvate flux in strain YK167 that only produces LDH and PDH* and not PFL activity was also directed towards LDH and not PDH* (Fig. 3B) and the PDH activity of this culture was only slightly lower than the value obtained with strain PMD23 (Table 2). However, strain YK167 extracts contained about 2.7-times higher level of LDH activity compared to strain PMD23 and this higher LDH activity could account for the flux through LDH over PDH* at the pyruvate node. In agreement with this higher LDH activity, the \( ldhA \) mRNA level of strain YK167 was found to be 2.5-fold higher than that of strain of PMD23. These results indicate that in the presence of both LDH and PDH*, LDH is the preferred physiological pathway of pyruvate metabolism in anaerobic \( E. \ coli \) for redox maintenance.

The rate of ethanol production (20.5 mmoles h\(^{-1}\) (g CDW)\(^{-1}\)) by strain QZ3 lacking LDH activity was the same as formate production rate (20.6 mmoles h\(^{-1}\) (g CDW)\(^{-1}\)) during the growth phase (Fig. 3C). Acetate production during this phase of growth was only 1.2 mmoles h\(^{-1}\) (g CDW)\(^{-1}\). Strain QZ3 also produced significant amount of pyruvate (10.6 mmoles h\(^{-1}\) (g CDW)\(^{-1}\)). The reducing equivalents (NADH) generated during pyruvate production and secretion was apparently used to reduce acetyl-CoA produced by PFL to ethanol accounting for the equimolar ethanol and formate in the medium. These results suggest that PDH* is not contributing to pyruvate flux in strain QZ3 during this initial phase of growth. Only when the culture reached the stationary phase (after about 10 h) PDH contribution to pyruvate flux can be detected based on the ratios of acetate, ethanol and formate production rates; rate of specific productivity for acetate, ethanol and formate are 1.1, 3.6 and 2.1 mmoles h\(^{-1}\) (g CDW)\(^{-1}\).

Although the initial growth rates of all three strains were comparable, significant amount
of glucose remained in the medium of strain QZ3 even after 116 hours of incubation in contrast to the other two strains with LDH activity (strains QZ2 and YK167; Fig. 3A, 3B). This lower rate of glucose consumption of strain QZ3 compared to strain QZ2 suggests that PDH* and PFL are rate-limiting at the pyruvate node. This is in agreement with the observation that pyruvate was detected in the broth of only strain QZ3 among the three strains tested (Fig. 3). However, the PDH* activity of strain QZ3 was comparable (about 0.4 unit mg protein\(^{-1}\) in crude extracts) to wild type strain PMD23 (Table 2), a characteristic that may be related to higher accumulation of pyruvate, a known activator of pdh operon and secretion by strain QZ3.

**Pyruvate distribution in a steady-state culture.** Anaerobic batch cultures of *E. coli* in which the growth rate is constantly changing may not be sensitive enough to measure flux through PDH*. To overcome this limitation, various mutants lacking one or more of the enzymes at the pyruvate node were cultured in a chemostat operating at a dilution rate of 0.1 h\(^{-1}\). In these experiments a rich medium was used to minimize the amount of carbon that is diverted to biosynthesis.

**Strains with active LDH and PFL.** The biochemistry framework depicted in Fig. 2 was used to determine the flux values using a steady-state glucose-limited chemostat culture. The detailed flux distributions of these cultures are presented in Table 3. The cell mass of strain PMD23 that is wild type for fermentation except for the absence of formate hydrogen-lyase (FHL) activity *(hypF)* stabilized at about 1.8 g/L and the specific glucose consumption rate was about 5.8 mmol h\(^{-1}\) (g CDW\(^{-1}\)). The major fermentation products of strain PMD23 were acetate, lactate and ethanol with the carbon flux ratio to these products at about 1:1:1 (J7, J10 and J11; Tables 3 & 4).
Carbon flux to formate was the sum of flux to acetate and ethanol, as expected. Production of lactate indicates that this culture is apparently ATP limited and not limited for reductant since the rich medium provided the needed precursors for biosynthesis of macromolecules. The additional reductant generated due to an increase in carbon flux for the production of ATP is apparently channeled to lactate to maintain redox balance. This is about 30% of the carbon flux at the pyruvate node (Table 4). The higher than expected flux to lactate in strain W3110 derivatives compared to strain MC4100 derivative reported by Yang et al. (42) could reflect the significant genotype differences of the two strains (25).

Introducing an active PDH into strain PMD23 through the \textit{lpd101} mutation (PDH*; strain QZ2) did not significantly alter the glucose flux through the three enzymes (Tables 3 & 4). This lack of carbon flux through PDH* in a strain with active LDH is in agreement with the batch culture experiments (Fig. 3A) and suggests that the LDH is the preferred enzyme in \textit{E. coli} to support redox balance.

**Strain QZ3 lacking LDH activity (PFL* and PDH**). Deleting the \textit{ldhA} gene (strain QZ3) forced about 50% of the pyruvate carbon through PDH* (Tables 3 & 4). This can be seen by the higher ethanol production rate over that of acetate. Since the PFL-based reactions yield an acetate to ethanol ratio of 1.0, the additional ethanol produced by stain QZ3 is expected to come from the PDH*/ADH pathway (Fig. 1). In strain QZ3, the amount of carbon diverted to PDH* was slightly higher than the amount of carbon flux through LDH to lactate in strain QZ2 (3.9 vs 2.9 mmoles h$^{-1}$ (g CDW)$^{-1}$, respectively). Surprisingly, the pyruvate flux through PFL was about 35% lower in strain QZ3 compared to strain QZ2 although the steady state cell mass of the two
cultures in the chemostat was comparable (about 2.0 g cell dry weight L\(^{-1}\)). The reason for this decline in pyruvate flux through PFL in a strain lacking LDH is not clear although the lower apparent pyruvate \(K_m\) for PDH* (0.4 mM vs 2.0 mM for PFL) may be a contributing factor.

Strain QZ3 also produced pyruvate as a fermentation product as seen with batch fermentations (Fig. 3C) suggesting that pyruvate conversion to acetyl-CoA by PFL and PDH* is rate-limiting in this strain. Even with this potential limitation, the steady state cell mass of the culture (about 2 g/L) and calculated \(Y_{ATP}\) values for strains QZ2 and QZ3 were comparable (\(Y_{ATP}\) values of 12.9 and 13.6, respectively) (Table 2) and similar to reported \(Y_{ATP}\) values for \(E.\ coli\) (16, 22, 24, 42). These results show that PDH* effectively competes with PFL for pyruvate even though PDH* activity requires NAD\(^+\). This is in contrast to the inability of PDH* to compete for pyruvate when all three enzymes are present (strain QZ2) and confirms that LDH pathway outcompetes PDH* for pyruvate, in support of maintaining redox balance in \(E.\ coli\) strain W3110.

**Strain YK167 lacking PFL activity (LDH\(^+\) and PDH\(^+\)).** A mutant that lacks PFL activity (YK167) while still retaining LDH and PDH* activities had an almost 50% increase in glucose consumption and flux compared to the strains with active PFL (strains QZ2 and QZ3) (Table 3). Apparently, the higher glucose flux to lactate is geared to ATP production at the glycolysis step compensating for the absence of post-pyruvate ATP production. This is comparable to similar increase in glucose flux reported for a \(pfl\) or \(ackA-pta\) mutant with native PDH complex over their corresponding parent strains (39, 42). Although PDH* was unable to compete with LDH for pyruvate in strain QZ2, in strain YK167, flux through PDH* was about 33% of the total pyruvate
(Tables 3 & 4). This flux through PDH* in strain YK167 was comparable to the observed PDH* flux in strain QZ3 lacking LDH activity (about 4 mmol·h⁻¹·(g cell dry weight)⁻¹). In the absence of PFL, PDH* is the only other enzyme that can generate acetyl-CoA that can be converted to acetate with associated ATP production. However, the NADH generated during pyruvate oxidation by PDH* is expected to limit the amount of ATP that can be produced by the PDH*, PTA, ACK pathway due to the need for redox balance. The rate of PDH*-dependent acetate production may be dictated by the rate at which the growing culture can utilize NADH. Although the growth rate of strain YK167 was comparable to the wild type strains in batch cultures, the Y_{ATP} for this strain in the chemostat was a significantly lower 6.9, compared to values that are higher than 10.0 for the strains with active PFL (Table 3). This is unexpected and is different from the Y_{ATP} value of 15.2 reported for an ackA, pta mutant of a strain MC4100 derivative (42). This difference in Y_{ATP} between the pfl mutant, strain YK167, and the ackA, pta mutant of Yang et al. (42), could result from the genotype differences of the two strains of E. coli K-12; strain W3110 used in this study (ATCC 27325) is believed to be nearly wild type (ATCC) while strain MC4100 has multiple alterations and deletions (25) with unknown physiological consequences. Strain MC4100 is also reported to have a lower level of FNR, the anaerobic control protein, compared to another wild type strain MG1655 (31). The potential effect of lower level of FNR in modulating the Y_{ATP} of an anaerobic culture is not known and needs investigation.

The Y_{ATP} calculations were based on predicted ATP yield on the fermentation products from the chemostat cultures and does not take into account any loss of ATP through a futile cycle (21, 26, 30). It is possible that in the absence of PFL, a futile cycle is draining ATP from glycolytic
reactions in strain YK167. One possible ATP loss could be an alternate pathway for phosphoenolpyruvate conversion to pyruvate through oxaloacetate and malate catalyzed by PEP-carboxylase, malate dehydrogenase and NAD^+-dependent malate dehydrogenase (decarboxylating). Due to the absence of PFL, and accumulation of pyruvate, the PEP-carboxylase may initiate this alternate pathway with the loss of ATP. This pathway is redox neutral and is not expected to alter the redox balance. It is also possible that in the absence of PFL, transport and assimilation of amino acids and other components from the medium may be limiting the growth leading to an ATP excess condition, initiating potential futile energy cycle(s).

**Strain SE2382 lacking both LDH and PFL activities (PDH*).** Glucose flux in Strain SE2382 that lacks both PFL and LDH activities was slightly lower than strain QZ3 (Δldh) but was reduced by about 50% compared to strain YK167 (ldh^+ but ΔpflB) (Table 2). Pyruvate flux through the PDH* in strains YK167 and SE2382 was similar suggesting that the loss of LDH activity in strain SE2382 due to mutation is the primary reason for the decrease in glucose flux. As expected, the lower rate of glycolysis in strain SE2382 led to lower cell mass in steady state cultures. The calculated Y_{ATP} values for strains SE2382 and YK167 were comparable and were about 50% of the value for PFL-positive strain QZ3. These results show that the absence of PFL is responsible for the lower cell yield of the pfl mutants in steady state cultures apparently due to lower ATP yield irrespective of the presence or absence of LDH activity.

Strain SE2382 also produced pyruvate as a product suggesting that the rate-limiting step in glucose fermentation is PDH* activity. In addition, strain SE2382 also produced lactate, probably through the methylglyoxal pathway indicating an accumulation of
glyceraldehyde-3-phosphate and associated dihydroxyacetone-3-phosphate, the substrate for methylglyoxal synthase, the starting point for LDH-independent lactate production. Pyruvate or lactate was not detected in the broth of strain YK1 that carries additional mutations in the PdhR and the intergenic region between pdhR and aceE (Table 2) (18). These additional mutations increased the expression of PDH operon by about 4-fold compared to strain SE2382 (aceE mRNA level of 2.2 vs 9.4 pg µl$^{-1}$ of total RNA for strains SE2382 and YK1, respectively) and flux through PDH* by about 1.75-fold. Besides the absence of lactate and pyruvate in the broth and higher ethanol production rate by strain YK1, there was no other significant difference between strains SE2382 and YK1 in the chemostat cultures (Tables 3 & 4). The calculated $Y_{ATP}$ value for strain YK1 (4.9) was the lowest of the strains investigated.

It is known that flux through PFL would lead to ATP production that is essential for increasing growth rate of an anaerobic culture (13, 19, 39). Under the redox balance condition, the reactions in Fig. 2 can be used to maximize ATP production with linear optimization. The flux distributions in the mutant QZ2 were calculated to be almost the same with the calculated ATP maximized fluxes. There is no flux to PDH. When the flux to PDH was increased, the generated ATP from glucose actually decreased. The genome level flux balance model also gave the same results (28), upon setting the optimized objective as the maximum biomass generation. These results suggest that the cell prefers the PFL pathway over the PDH*, probably due to the ATP yield.
CONCLUSION

Flux through LDH or PDH* only serves to maintain redox balance in the wild type and the low flux to PDH* (about 2%) in the “wild type” E. coli that favors LDH is unexpected considering the apparent Km values for pyruvate for the three competing enzymes. Although PDH requires NAD+ for activity, the apparent Km for NAD+ for this enzyme is 70 µM compared to NADH apparent Km of 200 µM for LDH (18, 37). The reported intracellular NAD+ concentration in E. coli grown anaerobically in glucose-limited chemostat varies between 1 and 2.5 mM depending on the strain (8), a concentration that is significantly higher than the apparent NAD+ Km for PDH* (18). Considering the highest reported [NADH]/[NAD+] ratio of an anaerobic E. coli is less than 1.0 (8), kinetically, PDH* would be the preferred enzyme at the pyruvate node. However, in the presence of LDH and PFL, flux through PDH* is negligible (strain QZ2; Table 3) indicating that some other factor(s) besides enzyme kinetics plays a role in distributing pyruvate among the three enzymes. In strain QZ2, the level of PDH complex in cell extracts was about 0.14 unit mg protein−1, a reduction in activity of about 70% compared to strain QZ3 and “wild type” strain PMD23 (about 0.4 unit mg protein−1). Although strain QZ2 had a lower PDH activity compared to strain PMD23, the ratio of LDH to PDH* activities in the extract of these two strains was about 3.0 and may not account for the significant difference in pyruvate flux through PDH*. However, it should be noted that besides the kinetic constants, the intracellular enzyme concentration, level of various regulatory metabolites, substrate and product concentrations, etc. do play a significant role in the flux through a specific enzyme and a combination of these factors may divert pyruvate carbon to LDH. It is interesting to note that the
high glucose flux in strain YK167 lacking PFL activity was supported by elevating the LDH activity and not by increasing flux through PDH* (Table 2 & 3) although PDH* can support redox balance. These results suggest a complex physiological interaction among the three pathways at the pyruvate node that includes control at the genetic level.

PDH complex is highly regulated by various metabolic intermediates including pyruvate (7, 12, 33, 34) and accumulation of some of the effectors in the cell may have a negative effect on the activity of PDH*. Although this is possible, flux through PDH* in mutant strains lacking either PFL or LDH or both argues against complete inhibition of PDH activity by metabolic inhibitors. Apparently, there are other unknown intricate physiological factor(s) that regulate flux at the pyruvate node towards lactate production for redox balance. One possibility is that the ADH is rate-limiting and the accumulation of acetyl-CoA is inhibitory to PDH* activity (33). In strains PMD23, QZ2, YK167 and SE2382, the overall flux through ADH (step J11, Table 3 and Fig. 2) is an almost constant value of about 3 mmol. h\(^{-1}\). (g cell dry weight)\(^{-1}\). Although flux through reaction J11 (Fig. 2) increased to 5.8 (mmol. h\(^{-1}\). (g cell dry weight)\(^{-1}\)) in strain QZ3 (\(\Delta ldh, lpd101\)) the level of ADH activity in the extract of this strain was only slightly higher (0.05 unit mg protein\(^{-1}\)) than that of strains PMD23 and QZ2 (0.04 unit mg protein\(^{-1}\)). These physiological and biochemical results suggest that ADH activity may not be limiting flux through PDH* in strain QZ2. This is further confirmed by the absence of increased flux through PDH* even after increasing the expression of *adhE* gene from a plasmid in strain QZ2 (data not presented).

Pyruvate is reported to function as an allosteric activator of LDH and this may favor
pyruvate flux through LDH (37). Pyruvate is also a known activator of PDH operon expression (27) and thus pyruvate may not provide a unique advantage for flux through one or the other enzyme complex.

The results presented above suggest that the mechanism that splits pyruvate among the three available pathways (PFL, LDH and PDH*) is apparently determined by energy requirement and redox balance constraints. Under anaerobic conditions, the cell prefers PFL due to the energy supply and LDH for redox balance maintenance even in the presence of an active PDH complex. The lack of flux through PDH* in a strain with all three enzymes could result from a reluctance of the cell to elevate the level of NADH and further increase the [NADH]/[NAD+] ratio although this NADH can be reoxidized in subsequent ADH-dependent reactions. An understanding of the controls at the pyruvate node as well as the potential function of futile cycle(s) in the absence of PFL can provide unique insights at this critical pyruvate node towards rational metabolic engineering of *E. coli* and related bacteria for production of various biobased products including ethanol at high rate and yield.

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steady-state internal redox state (NADH/NAD) reflects the external redox state and is correlated with catabolic adaptation in *Escherichia coli*. J. Bacteriol. 181:2351-2357.


Table 1. Bacterial strains used in this study

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<th>Strain</th>
<th>Relevant genotype</th>
<th>Source or Reference</th>
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<td>ATCC 27325</td>
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<tr>
<td>AH218</td>
<td>BW25113 Δ(focA-pflB)-FRT-Km-FRT</td>
<td>(13)</td>
</tr>
<tr>
<td>AH240</td>
<td>Δ(focA-pflB)-FRT-Km-FRT</td>
<td>W3110 x P1 (AH218)</td>
</tr>
<tr>
<td>AH241</td>
<td>ΔldhA</td>
<td>(17)</td>
</tr>
<tr>
<td>BW25113</td>
<td>lacF, rrnB_{T_{14}}, ΔlacZ_{6016}, hsdR_{514}, araBAD_{AH33}, ΔrhaBAD_{LD78}</td>
<td>(6)</td>
</tr>
<tr>
<td>PMD23</td>
<td>ΔhypF-FRT-Km-FRT</td>
<td>(15)</td>
</tr>
<tr>
<td>SE2378</td>
<td>lpd101, ΔldhA, Δ(focA-pflB)-FRT-Km-FRT, pdhR</td>
<td>(17)</td>
</tr>
<tr>
<td>SE2382</td>
<td>lpd101, ΔldhA, Δ(focA-pflB)-FRT-Km-FRT</td>
<td>(18)</td>
</tr>
<tr>
<td>YK1</td>
<td>lpd101, ΔldhA, Δ(focA-pflB)-FRT, pdhR, Km\textsuperscript{5}</td>
<td>SE2378, Km\textsuperscript{5}</td>
</tr>
<tr>
<td>YK98</td>
<td>BW25113 Δlpd-FRT-Km-FRT</td>
<td>This study</td>
</tr>
<tr>
<td>YK134</td>
<td>Δlpd-FRT-Km-FRT</td>
<td>W3110 x P1 (YK98)</td>
</tr>
<tr>
<td>YK142</td>
<td>lpd101</td>
<td>YK134 x P1 (SE2382)</td>
</tr>
<tr>
<td>YK143</td>
<td>ΔldhA, Δlpd- FRT-Km-FRT</td>
<td>AH241 x P1 (YK98)</td>
</tr>
<tr>
<td>YK167</td>
<td>lpd101, Δ(focA-pflB)- FRT-Km-FRT</td>
<td>YK142 x P1 (AH240)</td>
</tr>
<tr>
<td>QZ1</td>
<td>ΔldhA, lpd101</td>
<td>YK143 x P1 (YK142)</td>
</tr>
<tr>
<td>QZ2</td>
<td>lpd101, ΔhypF- FRT-Km-FRT</td>
<td>YK142 x P1 (PMD23)</td>
</tr>
<tr>
<td>QZ3</td>
<td>ΔldhA, lpd101, ΔhypF- FRT-Km-FRT</td>
<td>QZ1 x P1 (PMD23)</td>
</tr>
</tbody>
</table>

All strains are derivatives of *E. coli* K-12 strain W3110, a nearly wild type strain deposited by J. Lederberg, unless noted otherwise (BW25113 and YK98).
Enzyme activities were determined in extracts of cells harvested from fermenter cultures in mid-exponential phase of growth. PFL, pyruvate formate-lyase; LDH, fermentative lactate dehydrogenase; PDH*, pyruvate dehydrogenase complex with a mutation in dihydrolipoamide dehydrogenase; ADH, alcohol dehydrogenase. See Methods section for assay conditions and other details.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Enzymes Present</th>
<th>Enzyme Activity (µmoles min⁻¹ mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMD23</td>
<td>wild type</td>
<td>PFL; LDH</td>
<td>1.52 0.46 0.04</td>
</tr>
<tr>
<td>QZ2</td>
<td>lpd101</td>
<td>PFL; LDH; PDH*</td>
<td>0.41 0.14 0.05</td>
</tr>
<tr>
<td>QZ3</td>
<td>lpd101, ldhA</td>
<td>PFL; PDH*</td>
<td>0.01 0.44 0.04</td>
</tr>
<tr>
<td>YK167</td>
<td>lpd101, pflB</td>
<td>LDH; PDH*</td>
<td>4.17 0.32 0.06</td>
</tr>
</tbody>
</table>

Table 2. Enzyme activities of select mutants with alteration in the pyruvate node
Table 3. Metabolic flux distributions of different mutant strains of *E. coli* affected at the pyruvate node

<table>
<thead>
<tr>
<th>Flux to:</th>
<th>PMD23</th>
<th>QZ2</th>
<th>QZ3</th>
<th>YK167</th>
<th>SE2382</th>
<th>YK1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G-6-P</td>
<td>G-3-P</td>
<td>PEP</td>
<td>PYR</td>
<td>SUCC</td>
<td>Formate</td>
</tr>
<tr>
<td>J1</td>
<td>5.85</td>
<td>4.87</td>
<td>9.74</td>
<td>3.58</td>
<td>0.30</td>
<td>6.60</td>
</tr>
<tr>
<td>J2</td>
<td>5.00</td>
<td>4.70</td>
<td>9.40</td>
<td>4.10</td>
<td>0.30</td>
<td>6.00</td>
</tr>
<tr>
<td>J6</td>
<td>4.45</td>
<td>4.29</td>
<td>8.44</td>
<td>3.79</td>
<td>0.20</td>
<td>3.81</td>
</tr>
<tr>
<td>J7</td>
<td>7.30</td>
<td>7.26</td>
<td>14.53</td>
<td>6.31</td>
<td>0.91</td>
<td>0.00</td>
</tr>
<tr>
<td>J8</td>
<td>3.62</td>
<td>3.38</td>
<td>2.20</td>
<td>2.00</td>
<td>0.38</td>
<td>0.00</td>
</tr>
<tr>
<td>J9</td>
<td>4.06</td>
<td>3.86</td>
<td>3.33</td>
<td>3.33</td>
<td>0.33</td>
<td>0.00</td>
</tr>
<tr>
<td>J10</td>
<td>J11</td>
<td>J12</td>
<td>J13*</td>
<td>NADH</td>
<td>NADH</td>
<td>Cell Dry</td>
</tr>
<tr>
<td>J10</td>
<td>Acetate</td>
<td>Ethanol</td>
<td>Pyruvate</td>
<td>Formation</td>
<td>consumption</td>
<td>g/L</td>
</tr>
<tr>
<td>J10</td>
<td>3.58</td>
<td>3.04</td>
<td>0.00</td>
<td>9.76</td>
<td>9.50</td>
<td>1.84</td>
</tr>
<tr>
<td>J11</td>
<td>3.37</td>
<td>2.79</td>
<td>0.01</td>
<td>9.56</td>
<td>9.11</td>
<td>2.18</td>
</tr>
<tr>
<td>J12</td>
<td>1.93</td>
<td>5.78</td>
<td>0.52</td>
<td>12.34</td>
<td>12.12</td>
<td>1.93</td>
</tr>
<tr>
<td>J13*</td>
<td>1.01</td>
<td>3.30</td>
<td>0.43</td>
<td>18.83</td>
<td>17.30</td>
<td>1.50</td>
</tr>
<tr>
<td>J13*</td>
<td>0.92</td>
<td>3.31</td>
<td>0.43</td>
<td>10.44</td>
<td>7.94</td>
<td>0.69</td>
</tr>
<tr>
<td>J13*</td>
<td>1.13</td>
<td>6.26</td>
<td>1.59</td>
<td>15.11</td>
<td>13.19</td>
<td>0.60</td>
</tr>
<tr>
<td>J13*</td>
<td>0.00</td>
<td>0.56</td>
<td>0.56</td>
<td>15.11</td>
<td>13.19</td>
<td>0.60</td>
</tr>
<tr>
<td>NADH consumption</td>
<td>1.00</td>
<td>0.01</td>
<td>0.00</td>
<td>10.44</td>
<td>15.11</td>
<td>0.60</td>
</tr>
<tr>
<td>Cell Dry</td>
<td>1.13</td>
<td>6.26</td>
<td>1.59</td>
<td>15.11</td>
<td>13.19</td>
<td>0.60</td>
</tr>
<tr>
<td>Cell Dry</td>
<td>0.00</td>
<td>0.56</td>
<td>0.56</td>
<td>15.11</td>
<td>13.19</td>
<td>0.60</td>
</tr>
</tbody>
</table>

All cultures were grown anaerobically in a chemostat with glucose limitation at a dilution rate of 0.1 h\(^{-1}\) as indicated in the Methods section. Flux unit is mmol h\(^{-1}\) (g CDW\(^{-1}\)). Reactions J1, J6, J7, J8, J10, J11, J12 and J13 were directly measured while the other reaction rates were computed.

* The lactate produced by the *ldhA* deletion mutants (strains QZ3 and SE2382) is assumed to be derived from the methylglyoxal (J13) pathway and in strains with LDH activity, lactate produced by the methylglyoxal pathway (J13) was considered to be negligible.
Table 4. Anaerobic flux ratios at the pyruvate and acetyl-CoA branch points in select mutants with a mutation in PDH, LDH and/or PFL

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzymes†</th>
<th>Pyruvate branch</th>
<th>Acetyl-CoA branch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PFL</td>
<td>PDH</td>
</tr>
<tr>
<td>PMD23</td>
<td>PFL, LDH</td>
<td>0.70</td>
<td>0.002</td>
</tr>
<tr>
<td>QZ2</td>
<td>PFL, LDH, PDH*</td>
<td>0.66</td>
<td>0.01</td>
</tr>
<tr>
<td>QZ3</td>
<td>PFL, PDH*</td>
<td>0.48</td>
<td>0.50</td>
</tr>
<tr>
<td>YK167</td>
<td>LDH, PDH*</td>
<td>0.00</td>
<td>0.33</td>
</tr>
<tr>
<td>SE2382</td>
<td>PDH*</td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>YK1</td>
<td>PDH*</td>
<td>0.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

† Represents the enzymes that are expected to be functional at the pyruvate node in the indicated strains based on the genotypes, phenotypes and enzyme activities in the extracts.
Figure Legend

1. Anaerobic metabolic pathways of *E. coli* carrying *lpd101* mutation (PDH*).

2. Central metabolic pathways of *E. coli* used for flux analysis.

3. Although PDH* also produces acetyl-CoA (reaction J9), due to NADH production, most of this acetyl-CoA was assumed to only flow through reaction J11 to maintain redox balance.

4. Growth and fermentation characteristics of *E. coli* strains QZ2, YK167 and QZ3 with different enzyme composition at the pyruvate node in pH-controlled batch cultures. See Methods section for details. Concentration of lactate and succinate in the broth of strain QZ3 fermentation at 60 h was 19 mM and 20 mM, respectively. Formate was not detected in the broth of strain YK167.
Fig. 1
½ Glucose
Pyruvate Lactate
PDH*
Acetate
ATP NADH
ADP + P
NAD+
NAD+NADH
2 NADH
2 NAD+
NAD+NADH
PFL
2 NADH
2 NAD+
Acetyl-CoA + Formate
LDH
ADH-E
ADH-EPTA
ACK
Ethanol
Acetyl-CoA
Ethanol
ADP + P
ATP
Glycolysis
CO2
Fig. 2

Glucose → Glucose-6-P → Glyceraldehyde-3-P → 2 PEP → Pyruvate

Dihydroxyacetone-P → Methylglyoxal

Lactate → Pyruvate → Pyruvate (out)

Formate → AC-CoA → Acetate → Ethanol

Biomass

Succinate

ADP + Pi → ATP → 2 NAD^+ → NAD^+ → NADH

on December 18, 2017 by guest
Fig. 3A
Fig. 3B

Strain YK167

(lpd101 pflB)

Glucose
Lactate
Growth
Ethanol
Succinate
Acetate

Time (h)

[Glucose] or [Products] (mM)

Growth (OD420 nm)
Fig. 3C