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A Novel Fermentation Pathway in an *Escherichia coli* Mutant Producing Succinic Acid, Acetic Acid, and Ethanol

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ABSTRACT

Escherichia coli strain NZN111, which is unable to grow fermentatively because of insertional inactivation of the genes encoding pyruvate: formate lyase and the fermentative lactate dehydrogenase, gave rise spontaneously to a chromosomal mutation that restored its ability to ferment glucose. The mutant strain, named AFP111, fermented glucose more slowly than did its wild-type ancestor, strain W1485, and generated a very different spectrum of products. AFP111 produced succinic acid, acetic acid, and ethanol in proportions of approx 2:1:1. Calculations of carbon and electron balances accounted fully for the observed products; 1 mol of glucose was converted to 1 mol of succinic acid and 0.5 mol each of acetic acid and ethanol. The data support the emergence in *E. coli* of a novel succinic acid:acetic acid:ethanol fermentation pathway.

Index Entries: *Escherichia coli*; fermentation; succinic acid.

INTRODUCTION

In microbial fermentations of organic growth substrates, energy-yielding oxidative reactions generate a pool of partially oxidized intermediates and a surplus of reduced cofactors *(1)*. The excess reductant generated, primarily NADH, must be dissipated for metabolism to continue, and in most cases this is accomplished by reducing the available organic intermediates to end products that are excreted into the medium. The nature and proportions of these end products vary widely *(1)*. *Escherichia coli* produces several end products from the fermentation of sugars,

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principally ethanol and acetic, formic, and lactic acids *(2,3)*. Under some conditions, formic acid is further metabolized to hydrogen and CO_2. Smaller amounts of succinic acid are also formed by carboxylation of phosphoenolpyruvate (PEP) and reductive reactions of the tricarboxylic acid (TCA) cycle. The ratio of these products varies, depending on the strain and the growth conditions, but is always adjusted to balance the overall metabolism by consuming exactly the reducing equivalents generated in glycolysis *(2)*.

Some fermentative organisms produce succinic acid in higher amounts. Certain propionic acid bacteria make large amounts of succinic acid by fermentation of lactate *(1)*. In other cases, succinic acid may be formed from glucose. At low pH, the obligate anaerobe *Anaerobiospirillum succiniciproducens* ferments glucose plus CO_2 to a mixture of succinic and acetic acids in a 2:1 molar ratio *(4)*. Recently, the strain *Bacterium* 130Z has been shown to produce succinic acid as its major fermentation product, along with smaller amounts of acetic, formic, propionic, and pyruvic acids *(5)*. Wild-type *E. coli*, on the other hand, consistently forms only small amounts of succinic acid under a wide variety of conditions.

The authors have previously shown that overexpression of PEP carboxylase, or, in the appropriate genetic background, of malic enzyme, can significantly increase *E. coli*'s production of succinic acid *(6,7)*. A spontaneous mutation in a nonfermenting strain of *E. coli* that restored its ability to ferment glucose was recently discovered by the authors. Succinic acid was the major product. In addition, substantial but lower amounts of acetic acid and ethanol were formed. Here the discovery of the mutant, the quantitative characterization of its fermentative metabolism, and aspects of electron balance that affect product distribution is described. The pathway constitutes the first known instance of a succinic acid:acetic acid:ethanol fermentation.

MATERIALS AND METHODS

Bacterial Strains and Culture Methods

Strains of *E. coli* (Table 1) were routinely cultured in Luria broth (LB) or M9 medium at 37°C *(8)*. Anaerobic minimal medium was supplemented with Fe, Se, Mo, and Mn *(9)*. For plates, anaerobic conditions were established by use of a GasPak (Becton Dickinson, Cockeysville, MD) and grown under a atmosphere of H_2:CO_2. Liquid anaerobic cultures were grown in stoppered serum tubes under an atmosphere of sterile, anaerobic CO_2 at 14 psi, which was established by use of a gassing manifold *(10)*. During the fermentation of higher concentrations of glucose in rich medium, the pH was controlled by the addition of solid $MgCO_3$ to the serum tubes. To monitor the growth of anaerobic cultures, cells were grown in serum tubes containing minimal medium without $MgCO_3$, and absorbances were moni-

Table 1
Bacterial Strains and Plasmids

Strain	Relevant markers	Source or ref.
W1485	F^+ wild type	CGSC 5024
FMJ123	W1485 *pfl*::Cam	Bunch et al. *(11)*
NZN111	FMJ123 *ldh*::Kan	Bunch et al. *(11)*
AFP111	Spontaneous mutant of NZN111	This work
pMDH14	*E. coli mdh* (A80P/R81Q) in pTRC99a (Pharmacia, Piscataway, NJ)	Boernke et al. *(12)*

tored with a Spectronic 20 (Bausch & Lomb, Rochester, NY). When appropriate, antibiotics were added at the following concentrations: ampicillin, 100 μg/mL; carbenicillin, 100 μg/mL; tetracycline, 10 μg/mL; kanamycin, 30 μg/mL; chloramphenicol, 30 μg/mL. Strain NZN111 *(11)*, containing a plasmid encoding a mutant malate dehydrogenase that possessed lactate dehydrogenase activity *(12)*, was cultured in sealed serum tubes in 10 mL of LB medium, supplemented with 100 μg/mL carbenicillin, 12 g/L glucose, 0.1 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG), and 0.5 g $MgCO_3$.

Analytical Methods and Enzyme Assays

Glucose consumption and product formation were determined by high-performance liquid chromatography (HPLC) using a Bio-Rad (Hercules, CA) Aminex HPX-87H column (7.8 × 300 mm) and a Shimadzu LC-10A chromatography system equipped with UV absorbance and refractive index detectors. The column was eluted isocratically with 5 m*M* H_2SO_4, and data were analyzed with an EZChrom™ data system (Scientific Software, San Ramon, CA). Glucose levels were also monitored enzymatically using the commercial kit from Stanbio, (San Antonio, TX).

Analysis of Products Formed from [1-^{13}C]-D-Glucose

Quantitative measurement of the products formed from [1-^{13}C]-D-glucose (99.5 atom %, Cambridge Stable Isotopes, Andover, MA) was achieved by nuclear magnetic resonance (NMR) performed in the presence of the chemical relaxation reagent, erbium chloride. Because erbium salts of the products and of bicarbonate precipitated at neutral pH, the samples were acidified and degassed prior to its addition. Following the anaerobic conversion of 18.3 g/L [1-^{13}C]-D-glucose in LB medium under the conditions described above, cultures were clarified by filtration and acidified by treatment with Dowex AG50W-X4 (H^+ form) (Bio-rad, Hercules, CA), to convert the bicarbonate present to CO_2. The Dowex was removed by filtration and the sample was degassed under vacuum to completely remove the CO_2. Erbium chloride ($ErCl_3 \cdot 6H_2O$) was added to a final concentration of 7.9 (±0.3) m*M*. Spectra were obtained with a Varian (Palo Alto, CA) Unity 400 spectrometer at a probe frequency of 100.577 MHz. Tran-

sients (300 to 1000) of spectral width 25,000 Hz were collected at ambient temperature, with an acquisition time of 0.6 s, a relaxation time of 25 s, and a pulse width of 22 μs (PW90 = 25 μs). Protons were decoupled during acquisition, and the field was locked on D_2O. Chemical shifts are reported in parts per million, relative to tetramethylsilane. Reference spectra of succinic acid, acetic acid, and ethanol were also obtained, and the chemical shifts observed were within 0.1 ppm of published values.

RESULTS

Selection of Mutant *E. coli* Producing Succinic Acid

Previous site-directed mutagenesis of *E. coli* malate dehydrogenase performed in our laboratory generated a series of mutants with activity toward pyruvate, instead of the natural substrate, oxaloacetate *(12)*. The lactate dehydrogenase activity of these mutants was low, but sufficient to complement a chromosomal inactivation in *E. coli* of the fermentative lactate dehydrogenase gene, as illustrated in Fig. 1. When the mutant malate dehydrogenase encoded by plasmid pMDH14 (Table 1) was expressed in the nonfermenting *E. coli* host NZN111, slow fermentative production of lactic acid occurred (Table 2).

Since fermentative growth in NZN111(pMDH14) appeared to be limited by the low lactate dehydrogenase activity of the mutant malate dehydrogenase, experiments in directed evolution in an attempt to select a variant enzyme with improved lactate dehydrogenase activity were initiated. After several cycles of subculturing without mutagenesis, accelerated glucose catabolism was detected but found that other products, in addition to lactic acid, were formed (Table 2). Succinic acid was the major product observed, as well as lactic acid, acetic acid, and ethanol. To establish the source of the additional products, single colonies were isolated anaerobically on LB plates containing glucose, as well as kanamycin and chloramphenicol, the antibiotics encoded by the cassettes used to inactivate *pfl* and *ldhA* in the construction of NZN111 *(11)*. Isolated colonies were restreaked twice in the absence of antibiotic, then evaluated for antibiotic sensitivity. All colonies were resistant to kanamycin and chloramphenicol, but sensitive to ampicillin, indicating that they arose from NZN111 and no longer possessed the plasmid pMDH14. The pattern of fermentation products generated by several colonies was evaluated and found to be identical, indicating that the colonies were probably siblings. Succinic acid was the major fermentation product formed, with lower amounts of acetic acid and ethanol (Table 2).

Characterization of Mutant Strain

The purified mutant, named AFP111, was compared to its ancestral strains. The pattern of fermentation products it generated was very different from those formed by NZN111, its immediate precursor, FMJ123, the

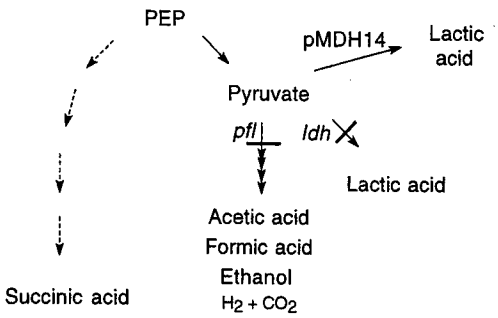

Fig. 1. Complementation of mutations in *E. coli* NZN111 by plasmid pMDH14. Inactivation (−) of the pyruvate:formate lyase and lactate dehydrogenase genes, *pfl* and *ldhA*, respectively, in strain NZN111 blocks the conversion of pyruvate and eliminates the ability to ferment glucose. Plasmid pMDH14 directs the expression of a mutant *E. coli* malate dehydrogenase with lactate dehydrogenase activity. Expression of this protein allowed NZN111 to metabolize glucose slowly by a homolactic acid fermentation.

Table 2
Emergence of Mutant Strain During Enzyme Selection Experiments[a]

	Glucose remaining and products formed (g/L)				
Strain	Glucose	Succinic acid	Lactic acid	Acetic acid	Ethanol
NZN111[b]	10.5	0.5	0	0	0
NZN111(pMDH14)[c]	3.8	0.8	5.7	0	0
Selection experiment[d]	0	5.8	3.0	2.6	1.7
Plasmid-free mutant[e]	0	8.4	0	2.2	1.4

[a] Products formed overnight from 12 g/L glucose in 10 mL of LB with $MgCO_3$ under an atmosphere of CO_2.

[b] Unable to ferment glucose. Pyruvate produced at 0.2–0.8 g/L (2–9 m*M*).

[c] pMDH14, which encodes *E. coli* MDH mutant with LDH activity, was induced with IPTG.

[d] Products formed by induced NZN111(pMDH14) after six cycles of subculturing.

[e] Named AFP111. Isolated as Amp^s, Kan^r, Cam^r single colonies.

parent of NZN111, which contains a functional *ldh* gene *(11)*, or W1485, the wild-type ancestor (Table 3). AFP111 fermented glucose to a mixture of succinic acid, acetic acid, and ethanol; FMJ123 metabolized glucose by a homolactic acid fermentation; and W1485 generated the typical spectrum of mixed acid fermentation products. NZN111 failed to ferment glucose and excreted pyruvate into the medium.

The anaerobic growth of AFP111 on glucose in minimal medium supplemented with casamino acids was compared to that of NZN111 and W1485 (Fig. 2). Strain NZN111 failed to grow, as reported earlier *(11,12)*;

Table 3
Products Formed by Strains in Lineage of Mutant AFP111[a]

Strain	Product (g/L)				
	Succinic acid	Lactic acid	Formic acid	Acetic acid	Ethanol
W1485	2.1	1.8	4.9	4.5	4.1
FMJ123	1.1	13.2	0.3	1.0	0.7
NZN111[b]	0.1	0.2	0.2	0.2	0.4
AFP111	11.3	0.6	0	3.9	2.3

[a] Products formed from 18.4 g/L glucose in 10 mL of LB broth incubated in sealed tubes under an atmosphere of CO_2 with 1 g $MgCO_3$ to maintain pH.
[b] Unable to ferment glucose. Pyruvate also produced at 1 g/L.

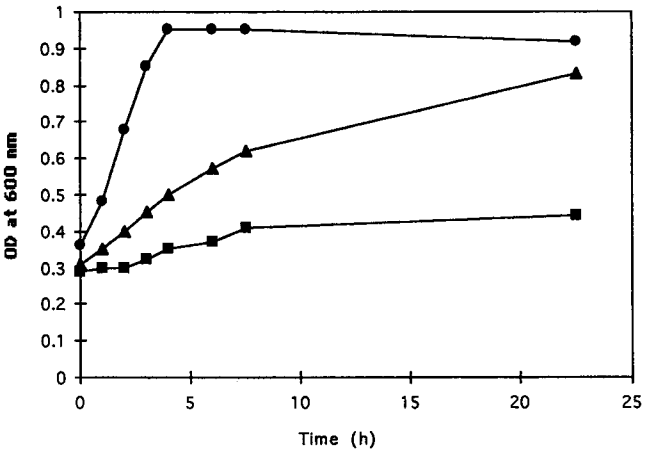

Fig. 2. Anaerobic growth of strains on glucose in minimal medium. Strains AFP111 (▲), its immediate parent NZN111 (■), and the wild-type ancestor W1485 (●) were grown anaerobically at 37°C, in 10 mL of M9 medium containing 2.9 g/L glucose, in sealed vials under an atmosphere of CO_2. Optical densities were measured with a Spectronic 20.

the small amount of growth observed was most likely caused by carryover of metabolites and oxygen in the inoculum. Strain AFP111 grew much more slowly than W1485, but eventually attained approximately the same final cell density.

Fermentation in minimal medium allowed quantitative evaluation of the fermentative metabolism of AFP111 (Table 4). Approximately 1 mol of succinic acid was generated per mol of glucose consumed. Acetic acid and ethanol were formed in lower, approximately equal amounts; in total, their amount approached 2 mol/mol of glucose. A trace of lactic acid was also formed. Analysis of the products in terms of carbon and electron

Table 4
Carbon and Electron Balances Glucose Fermentation by AFP111[a]

	Growth substrate and products		Carbon balance mol product/ 100 mol glucose	Electron balance mol NADH/ 100 mol glucose
	g/L	m*M*		
Glucose (substrate)	2.92	16		200
Succinic acid	1.90	16	99	−199
Lactic acid	0.13	1	9	−9
Acetic acid	0.47	8	48	48
Ethanol	0.29	6	39	−39
Cell mass[b]	0.16			
Total products[c]	2.95		195	1

[a] Products formed from 2.9 g/L glucose in M9 medium containing 1% casamino acids in sealed tubes under an atmosphere of CO_2.

[b] Cell mass estimated from the established relationship between cell mass and optical density.

balance indicated tight closure of the metabolic pathway. For the mixed acid fermentation pathway, a maximum of 200 mol of products (excluding formic acid) are anticipated per 100 mol of glucose *(1)*. The authors accounted for 195 mol as excreted products. Electron balance calculations, based here on reductions and oxidations required by established metabolic pathways for glucose fermentation in *E. coli*, supported tight coupling of reductant generation and consumption.

Metabolism of [1-^{13}C]-D-Glucose

To establish that all the products observed in rich medium arose from glucose, as opposed to other components of the medium, the products formed by AFP111 in the fermentation of [1-^{13}C]-D-glucose were analyzed. The chemical relaxation reagent erbium chloride was added to the treated broths, to permit quantitative integration of the resonances and determination of the relative abundances of the products. For clarity, a single set of assignments is used for both spectra (Fig. 3).

The spectrum of products generated by W1485 (Fig. 3A) reveals ethanol, lactic acid, acetic acid, and succinic acid methyl and methylene carbons (resonances a–d, respectively), the ethanol hydroxymethyl carbon (e), formic acid (f), and the acetic acid and succinic acid carboxyl carbons (g and h, respectively). No resonance for CO_2 is observed, because the samples were acidified and degassed prior to analysis. In the AFP111 spectrum (Fig. 3B), the increased abundance of the succinic acid resonances (d and h), relative to those in the W1485 spectrum, is apparent.

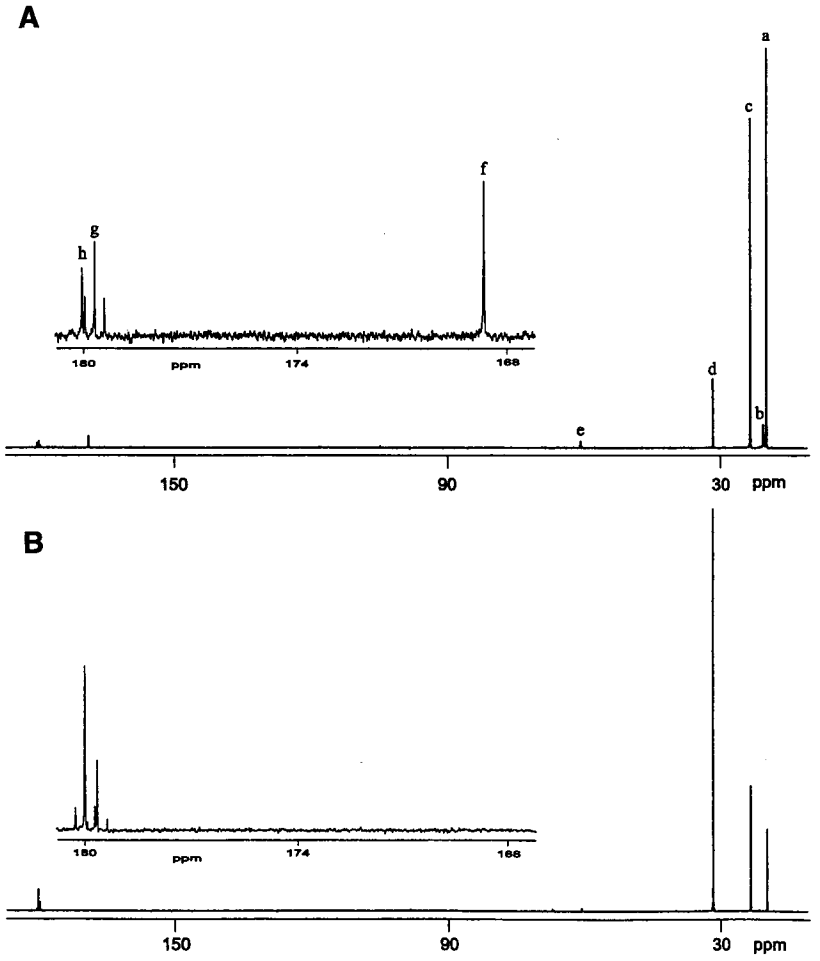

Fig. 3. ^{13}C-NMR spectra of fermentation products. Products of fermentation of 18.3 g/L of [1-^{13}C]-D-glucose by **(A)** W1485 and **(B)** AFP111. Peaks are assigned as follows: a, ethanol methyl carbon; b, lactic acid methyl carbon; c, acetic acid methyl carbon; d, succinic acid methylene carbon; e, ethanol hydroxymethyl carbon; f, formic acid carboxyl carbon; g, acetic acid carboxyl carbon; and h, succinic acid carboxyl carbon. The methyl and methylene carbon signals are much larger because of enrichment from [1-^{13}C]-D-glucose.

The relative abundance of the products formed from [1-^{13}C]-D-glucose can be determined from the integrated intensities of the alkyl resonances of each product—assuming that each species was formed from a single PEP. Calculation, in this manner, of the mole fraction of each product formed by AFP111 agreed reasonably well with the product distribution determined by HPLC analysis of the same experiment (Table 5). The estimates of succinic acid and ethanol are not fully in agreement, however; NMR estimates indicate slightly greater than stoichiometric formation of succinic acid from glucose, at the expense of reduced ethanol formation.

Table 5
Products Formed During Growth on [1-^{13}C]-Glucose in Rich Medium[a]

	Analysis by HPLC		Analysis by NMR	
Product	g/L	mol fraction[b]	Integrated intensity[c]	mol fraction[d]
Succinic acid	12.8	0.51	55.00	0.59
Lactic acid	0.2	0.01	0.53	0.01
Acetic acid	3.3	0.26	25.09	0.27
Ethanol	2.2	0.23	13.14	0.14
Total products	18.5	1.0	93.76	1.0

[a] Products formed from 18.3 g/L [1-^{13}C]-glucose (99 atom %) in 10 mL of LB broth incubated in sealed tubes under an atmosphere of CO_2 with 1 g $MgCO_3$ to maintain pH.

[b] Based on molarity of products formed, as calculated from g/L and mol wt.

[c] Integrated intensity of methyl carbon of lactic acid, acetic acid, and ethanol, and of methylene carbon of succinic acid.

[d] Based on summation of integrated intensities.

Possibly, a small amount of ethanol was lost by degassing during filtration of the sample following acidification and treatment with Dowex.

DISCUSSION

The results described above establish that the *E. coli* mutant AFP111 carries out a balanced fermentation of 1 mol glucose to 1 mol succinic acid, one-half mol of acetic acid, and approx one-half mol of ethanol (Fig. 4). These stoichiometries were obtained consistently, both in minimal supplemented with casamino acids and in rich media. They were substantiated by the distribution of ^{13}C derived from metabolism of [1-^{13}C]-D-glucose. The pathway represents the first example of an *E. coli* strain that produces succinic acid as its major fermentation product. Figure 4 satisfies the requirements of fermentations for both the balanced production and consumption of reducing equivalents and the generation of ATP. The ability of AFP111 is attributed to ferment glucose to a spontaneous chromosomal mutation that became enriched under the selective conditions of the directed evolution experiment.

The pathway can be envisioned as the conversion of glucose to two PEP, followed by reduction of one PEP to succinic acid, using the reducing power generated in glycolysis (two reductive steps are required for succinic acid formation), and conversion of the second PEP to an equal mixture of acetic acid and ethanol. The reducing equivalents generated in the oxidation of pyruvate are consumed in the reduction of acetylCoA to ethanol. The obligatory coupling of acetic acid and ethanol production documented earlier for *E. coli (13)* persists in AFP111.

The pathway proposed in Fig. 4 differs from a similar fermentation carried out under acidic conditions by *A. succiniciproducens*. This later path-

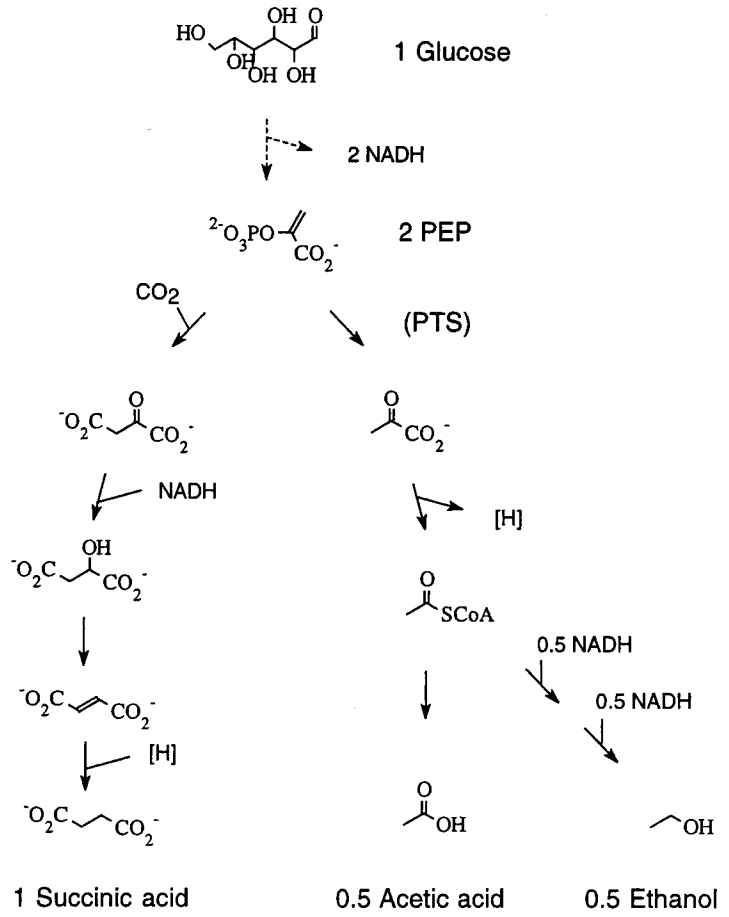

Fig. 4. Proposed fermentative pathway of AFP111. Because of the use of the PTS for the uptake of glucose, glycolysis in *E. coli* can be considered to yield one PEP, one pyruvate, and two reducing equivalents as NADH. Metabolism of PEP via the reductive arm of the TCA cycle would consume two reducing equivalents. Oxidation of pyruvate to acetylCoA would generate additional reducing equivalents, which can be consumed in the reduction of half of the acetylCoA to ethanol. The remaining acetylCoA would be hydrolyzed to acetic acid to complete the conversion of 1 mol glucose to 1 mol succinic acid, 0.5 mol acetic acid, and 0.5 mol ethanol.

way converts glucose to a 2:1 mixture of succinic and acetic acids *(4)*. It also satisfies the need for redox balance; two-thirds of the PEP formed in glycolysis is converted to succinic acid; oxidation of the remaining third to acetic acid provides the reducing equivalents needed for succinic acid formation. Such a pathway may not be possible in *E. coli* because of the use of the phosphotransferase system (PTS) for glucose uptake. Use of the PTS effectively mandates that half of the PEP generated glycolytically be converted to pyruvate *(14)*, which is then dissimilated to acetic acid and ethanol. Such a restriction would make the production of greater than stoichiometric amounts of succinic acid impossible.

Although the proposed pathway fits the data very well, and would be totally satisfactory for a less well-characterized organism, many aspects of it are not satisfactory in the face of the voluminous knowledge available for *E. coli*. Most obviously, the enzyme that catalyzes the conversion of pyruvate to acetylCoA needs to be determined. Pyruvate:formate lyase is not responsible, since this enzyme has been inactivated by disruption of the *pfl* gene. The locations of ^{13}C observed in the products generated from [1-^{13}C]-glucose suggest that conventional reactions of known enzymes are used, but the well-established regulation of these enzymes argues that they should not be operative under these conditions. Preliminary genetic mapping and transductions of genetic knock-outs of specific genes indicates that the mutation that created AFP111 was not in any of the structural genes of the pyruvate dehydrogenase complex. Neither was the mutation in the gene *fdx*, which encodes the ferrodoxin that participates in pyruvate: ferrodoxin oxidoreductase, nor in the global regulatory genes *arcB* or *fnr*.

Regardless of which enzyme catalyzes pyruvate conversion, the abundant production of succinic acid by AFP111 must be explained. Fermentation by AFP111 is slower than that of the wild type or of mutants lacking either lactate dehydrogenase or pyruvate:formate lyase alone, which metabolize glucose to a reduced spectrum of products. Possibly the slower metabolism in AFP111 results in the accumulation of both pyruvate and PEP to higher concentrations than normally occur in other strains, and thereby opens a metabolic channel normally of minor consequence in the presence of efficient competing pathways.

The authors are currently investigating the nature of the mutation, through a combination of genetic mapping, enzymatic analyses, and two-dimensional gel electrophoresis.

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