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Metabolic Flux Analysis of *Escherichia coli* Deficient in the Acetate Production Pathway and Expressing the *Bacillus subtilis* Acetolactate Synthase

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Several approaches to reduce acetate accumulation in *Escherichia coli* cultures have recently been reported. This reduction subsequently led to a significant enhancement in recombinant protein production. In those studies, metabolically engineered *E. coli* strains with reduced acetate synthesis rates were constructed through the modification of glucose uptake rate, the elimination of critical enzymes that are involved in the acetate formation pathways, and the redirection of carbon flux toward less inhibitory byproducts. In particular, it has been shown that strains carrying the *Bacillus subtilis* acetolactate synthase (ALS) gene not only produce less acetate but also have a higher ATP yield. Metabolic flux analysis of carbon flux distribution of the central metabolic pathways and at the pyruvate branch point revealed that this strain has the ability to channel excess pyruvate to the much less toxic compound, acetoin. The main focus of this study is the systematic analysis of the effects of small perturbations in the host's existing pathways on the redistribution of carbon fluxes. Specifically, a mutant with deleted acetate kinase (ACK) and acetyl phosphotransferase (PTA) was constructed and studied. Results from the metabolic analysis of carbon redistribution show the *ackA-pta* mutation will reduce acetate level at the expense of the growth rate. In addition, in the *ackA-pta* deficient strain a much higher lactate formation rate with simultaneously lower formate and ethanol synthesis rates was found. Expression of the *B. subtilis* ALS in *ackA-pta* mutants further reduces acetate levels while cell density similar to that of the parent strain is attained.

***Key Words*: *Escherichia coli*; metabolic flux analysis; *als*, *ackA*; *ptA*.**

INTRODUCTION

Escherichia coli is extensively used in industry as a host for recombinant protein production. The ease of genetic manipulation and wealth of available genetic information coupled with fast growth rate, standardized cultivation techniques, and cheap media are reasons for its popularity. However, conditions in high-density cultures result in accumulation of acetate and cessation of growth.

The reduction of acetate production is of primary concern in fermentation and recombinant protein production by

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E. coli. Acetate is produced in glucose fermentation by aerobic acetogenesis or through the Crabtree effect [1]. Acetate formation is due to excess influx of carbon from glucose that the cell is unable to utilize for biomass synthesis, leading to repression of the TCA cycle enzymes by glucose, or uncoupled metabolism [2]. Anaerobically, acetate production provides the ATP necessary for growth. Production of acetate represents a loss from carbon flux to cell growth as well as recombinant protein production. Acetate can also act as a liphophilic agent which can dissipate the pH component of proton motive force (PMF). Several previous reports have shown that short-chain fatty acids repress synthesis of DNA, RNA, protein, lipids, and peptidoglycans [3, 4]. These components of the cellular machinery are necessary for growth as well as protein production. Not suprisingly, the reduction of acetate accumulation has also been shown to enhance recombinant protein production [5].

For these reasons, several strategies for reducing acetate accumulation have been attempted. Industrial strategies tend toward modification of the external or environmental conditions through medium selection, glucose limitation, and aeration. The internal genotype of the host cell can also be altered. Some of these approaches include engineered strains with modification of the glucose uptake rate (*ptsG*, glucose phosphotransferase system) [6], redirection of the carbon flux to a less inhibitory byproduct (e.g., acetoin by *als*, acetolactate synthase [7] or ethanol through the PET operon) [8, 9], storage of excess carbon as glycogen [10] and elimination of the major acetate formation pathway (*ackA*, acetate kinase, *pta*, phosphotransacetylase) [11]. Each of these schemes has various effects on cell physiology and these observations point to the need for a more basic understanding of factors controlling the relative levels of acetate formation.

Metabolic engineering is a useful tool for providing precise perturbations of these physiological studies. The effects of these precise perturbations in the redistribution of fluxes can be studied using metabolic flux analysis (MFA) to provide insight into the effectiveness of a particular strategy as well as to suggest areas for additional modification [12–14]. MFA can also be used to gain a basic understanding of cell physiology.

Previously, our laboratory has used metabolic flux analysis in both batch and chemostat studies to determine the effect of the *Bacillus subtilis* acetolactate synthase (ALS) on the redistribution of flux in *E. coli* [14]. In this work, we focus on a study of the effects of an additional perturbation in the major acetate production pathway; the removal of the major acetate production pathway through mutations in *ackA* and *pta* was investigated. Finally, the combined effect of two acetate reduction systems, the heterologous expression of the *B. subtilis* ALS and the deletion of the *ackA-pta* pathway, was also examined.

TABLE 1

***Escherichia coli* Strains and Plasmids Used**

E. coli strains	Significant genotype	References
	Laboratory wild type, spontaneous	
GJT001	*cadR* mutant of MC4100	[26]
TA3516	*Δ(pta-ackA-hisQ-hisP)*	[15]
CAG18552	*zei*::Tn*10kan* at 49. min	[16]
YBS601	P1 CAG1852::TA3516	This paper
YBS125	P1 YBS601::GJT001	This paper
Plasmids		
pACYC184	Control-cloning vector	[27, 28]
pAAA215	*B. subtilis alsS*	[7, 29]

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Strains and plasmids used in this paper are listed in Table 1. To eliminate artifacts due to host strain variations, YBS125, an *ackA-pta* deficient derivative of GJT001 was constructed in this study using a two stage P1 phage transduction [6] to insert a characterized *ackA-pta* mutation into GJT001. In the first stage, the kanamycin cassette from CAG18552 was inserted near the *ackA* and *pta* genes of TA3516 [15, 16]. The kanamycin and *ackA-pta* mutations were cotransduced into GJT001. Sodium monofluroacetate (SMFA) and kanamycin plates were used for selection. SMFA plate was prepared using M9 salts supplemented with 0.44 g/L sodium citrate, 2.5 g/L pyruvate, 2.5 g/L SMFA, and 15.0 g/L agar [7]. Acetate kinase and phosphotransacetylase assays were conducted to verify the

TABLE 2A

Acetate Kinase and Phosphotransferase

Strains	PTA activity (U/mg protein)[a]	ACK activity (U/mg protein)[b]
GJT001 [laboratory wild type]	1.16	0.69
TA3516 [*ackA-pta*]	0.00	0.00
YBS601 [P1 CAG18522 (K_m^R)::TA3516(*ackA-pta*)]	0.00	0.00
YBS125 [P1 YBS601 (*ackA-pta*, K_m^R)::GJT001]	0.01	0.03

Note. Acetate kinase and phosphotransferase activities are assayed by method of Brown [30] and Rose *et al.* [31].

[a] One unit of PTA activity is defined as the amount of NADH formed in μmol per minute.

[b] One unit of ACK activity is defined as the amount of hydroxamate formed in μmol per minute.

TABLE 2B

Growth Characteristics

	Specific growth rate (h^{-1})
GJT001 [wild type]	1.41 ± 0.01
YBS125 [*ackA-pta*, K_m^R]	0.54 ± 0.01

Note. Specific activity is calculated for growth in LB supplemented with 20 g/L glucose in shake flasks maintained at 250 rpm and 37° C.

phenotypes (Table 2a). The growth characteristics for the strains GJT001 and YBS125 were compared using shake flask experiments in LB medium supplemented with 20 g/l of glucose. The results given in Table 2b are the averages of three experiments. The specific growth rate of the YBS125 strain is much slower than its parent strain, GJT001. However, the final optical density is more than 30% higher for YBS125. The more efficient utilization of available resources by the YBS125 strain is probably due to the slower growth rate and its lack of acetate formation capability.

Medium

Luria–Bertani broth (LB) medium supplemented with 20 g/L of glucose and 10 mg/L inositol was used for all cultivations. To reduce the initial lag time that occur under anaerobic conditions, 1 g/L $NaHCO_3$ was included. Tetracycline and kanamycin were added at concentrations of 25 mg/L and 50 mg/L to select for plasmid and chromosomally marked *ackA-pta* strains, respectively.

Cultivation Conditions

An anaerobic cultivation condition was chosen to avoid the uncertainties associated with the P/2e ratio of oxidative phosphorylation for ATP yield calculations. Under anaerobic growth without external electron acceptors such as nitrate or fumarate, energy generation occurs only through substrate level phophorylation. Chemostat cultivation was chosen over batch cultivation to provide better quality on the specific rate data.

The cells were cultivated in a 2.5-L bioreactor (New Brunswick Scientific, Bioflo III) initially with 1.3 L of medium during the aerobic batch stage and maintained at 1.25 L working volume for the anaerobic stage. The pH, temperature and agitation was maintained at 7.0, 32° C, and 250 rpm, respectively, with air flow at 3 L/min. A 1-h period with no gas flow allows the cells to acclimate to anaerobic conditions before the switch to anaerobic stage. A constant flow of nitrogen (10–15 ml/min) was maintained through the fermentor headspace to establish anaerobic condition. A dilution rate of 0.1 h^{-1} was maintained throughout the experiment. The continuous culture reaches steady state after 5 to 6 residence times. Samples were taken from an average of three samples taken at an interval of 5 to 10 h. Under these conditions, glucose is the limiting nutrient for the parent strain.

Analytical Technique

Cell density was monitored at 600 nm in a spectrophotometer and reported as cell dry weight. Cell dry weight was determined by collection of 100 ml of culture in an ice bath. The samples were centrifuged at 8,000*g* and 4° C for 10 min and dried in oven until constant weight.

Fermentation broth samples were collected and centrifuged as described above. The supernatant was filtered through a 0.45-μm syringe filter and stored chilled for further analysis. The glucose was enzymatically assayed (Sigma Hexokinase kit). Acetate, ethanol, and acetoin were quantified using a Varian 3000 gas chromatograph equipped with a porous polystyrene column (Poropak QS, Alltech) and a flame ionization detector (FID). Nitrogen was the carrier gas at 30 ml/min. The flame was maintained by H_2 and air at 30 ml/min and 300 ml/min, respectively. The injector and detector temperature were at 215 and 245° C, respectively. The column temperature profile used to resolve the peaks was initially at 115° C for 3 min followed by a 3.5° C/min ramp to 170° C and held at 170° C for 10 min. Other extracellular metabolites were quantified using an HPLC system (Waters) equipped with a cation-exchange column (HPX-87H, BioRad Labs) and a differential refractive index detector. A mobile phase of 2.5 mM H_2SO_4 solution at a 0.6 ml/min flowrate was used and the column was operated at 55° C.

Off-gas, specifically CO_2 and H_2, were analyzed off-line using the gas chromatograph equipped with a divinylbenzene porous polymer column (HayeSep DB, HayeSep) and a thermal conductivity detector. The column and injector were operated at 28° C with helium as the carrier gas at 30 ml/min. The detector and filament temperatures were set at 140 and 225° C, respectively.

RESULTS AND DISCUSSION

Metabolic Flux Analysis

Detailed construction of the metabolic flux analysis is described elsewhere [14]. Briefly, using the biochemistry framework depicted in Fig. 1, a metabolic matrix is constructed based on the law of mass conservation and on the pseudo-steady state hypothesis (PSSH) on the intracellular metabolites. The formulation resulted in a set of linear equations that can be expressed as a stochiometric matrix A of

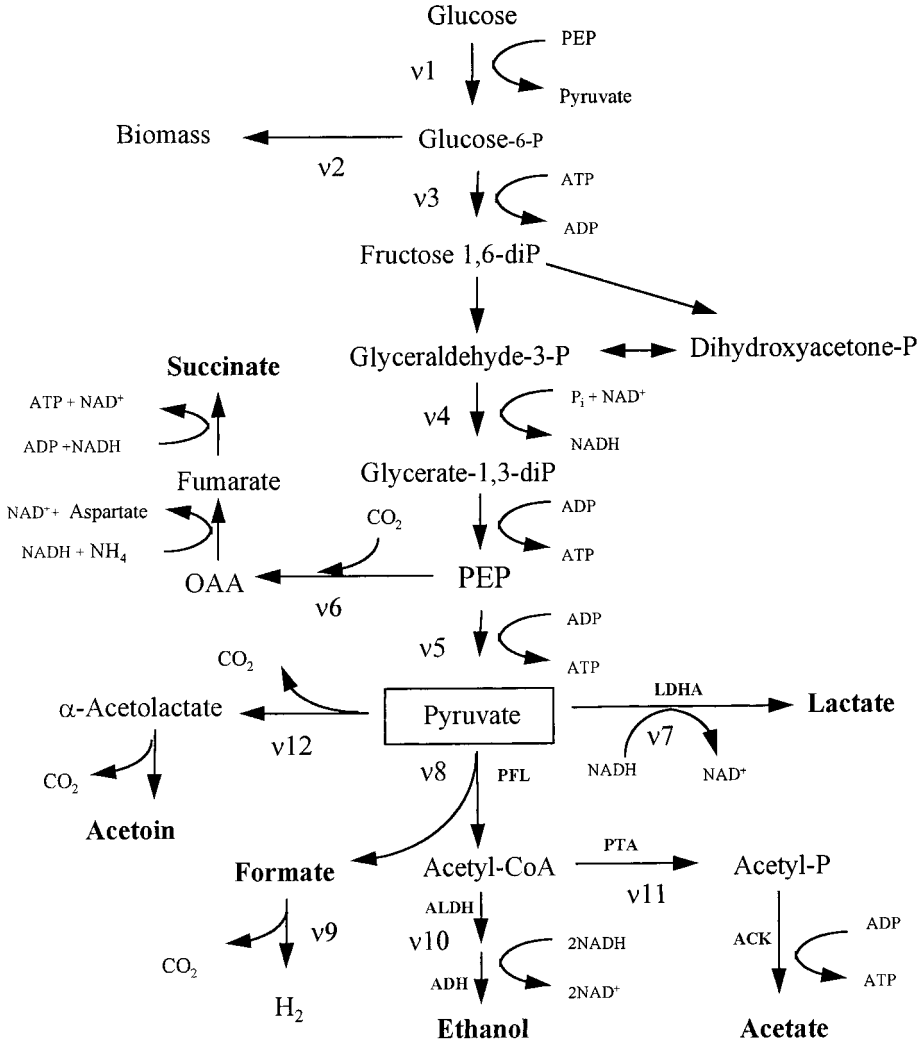

FIG. 1. Central anaerobic metabolic pathway of *E. coli.* The fermentation end-products include acetoin produced by cells containing the ALS plasmid. The fluxes through each pathway are designated v_1 through v_{12}.

dimension m by n with vectors for net accumulation, $\mathbf{r}$ $(m \times l)$ and metabolic flux, $\mathbf{v}$ $(n \times l)$. Theoretically, two different matrices can be formed using slightly different assumptions for the twelve metabolic fluxes (v_1 to v_{12} for this case). The first one based solely on the measured extracellular metabolites and PSSH balances on the intracellular intermediate metabolites resulting in a square matrix ($n = m = 12$). The addtional CO_2 and H_2 measurements produce an overdetermined system by which the flux calculation may be verified ($m = 14$). The solution requires the use of the least square fit. Table 3 lists the balances and fluxes involved in the analysis.

Figure 2 summarizes the cell dry weight and fermentation products for the strains GJT001 and YBS125. Pyruvate was measured but not detected for the parent or ALS containing strains. Low level (about 5 mM) of pyruvate was detected for YBS125. These extracellar byproducts along with the CO_2 and H_2 measurements were used in all flux calculation. The results of metabolic flux analysis for both approaches showed similar trends. Only the solution for the exact system ($n = m = 12$ case) is shown (Table 4).

Effect of ALS Expression

The expression of the biologically active *B. subtilis* ALS enzyme reduces the accumulation of acetate and lactate by about fourfold (Fig. 2). Carbon flux to acetate (v_{11}) decreased by more than 60% with a corresponding decrease in extracellular concentration of acetate.

The ratios of fluxes partitioned at the pyruvate and acetyl-CoA branch points are listed in Table 5. At the pyruvate branch point, partitioning to acetyl-CoA commits

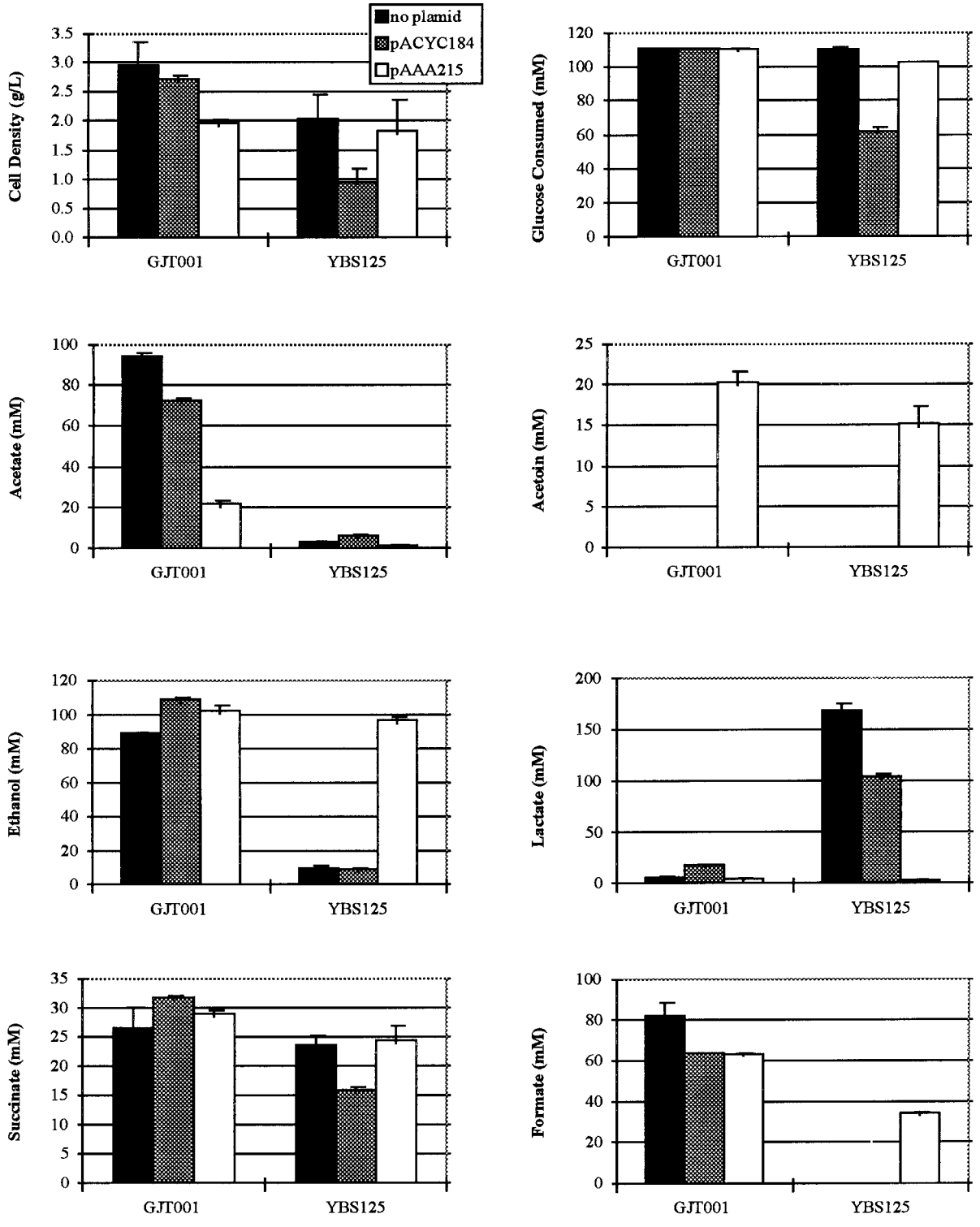

FIG. 2. Summary of chemostat experimental results. Anaerobic growth in LB supplemented with 20 g/L of glucose at 32° C at a constant dilution rate of 0.1 h^{-1}.

TABLE 3

Stoichiometric Flux Relationships for Anaerobic Growth

i[a]	Metabolite	C-atoms	O/R	Net conversion: $\mathbf{r}_i(t)$
1	Acetate	2	0	v_{11}
2	Acetoin	4	−2	v_{12}
3	Ethanol	2	−2	v_{10}
4	Formate	1	+1	$v_8 - v_9$
5	Glucose	6	0	v_1
6	Lactate	4	0	v_7
7	Succinate	4	+1	v_6
8	CO_2	1	+2	$-v_6 + v_9 + 2v_{12}$
9	H_2	0	−1	v_9
10	Acetyl-CoA			$v_8 - v_{10} - v_{11}$
11	Glucose 6-phosphate			$v_1 - v_2 - v_3$
12	G 3-phosphate			$2v_3 - v_4$
13	PEP[b]			$-v'_5 + v_4 - v_6$
14	Pyruvate			$v'_5 - v_7 - v_8 - 2v_{12}$
	NADH			$v_4 - 2v_6 - v_7 - 2v_{10}$
	ATP			$3v_3 + v_{11} - v_m$
	O/R			$-v_6 + v_8 - 2v_{10} + 2v_{12}$
	C to byproducts			$3v_6 + 4v_7 + v_8 + 2v_{10} + 2v_{11} + 6v_{12}$
	C to biomass[c]			v_2

[a] The metabolites 1–9 are to measurable quantities while 10–14 represent metabolic intermediates.
[b] $v'_5 = v_1 + v_5$ is the overall flux of PEP to pyruvate.
[c] Glucose not fermented to fermentation byproducts is assumed to be converted to biomass.

TABLE 4

Effect of *ackA-pta* Mutation and ALS Expression on Metabolic Flux Distribution for Anaerobic Chemostat Cultivation in Complex Media Supplemented with Glocuse

		GJT001 (wild type)			YBS125 (*ackA-pta*)		
Flux	to:	Host alone	Control plasmid	als plasmid	Host alone	Control plasmid	als plasmid
v_1	G-6-P	3.55	4.34	5.60	5.39	6.73	5.54
v_2	Biosyn.[a]	0.09	0.00	0.57	0.39	0.00	1.32
v_3	G-3-P	3.46	4.51	5.03	5.01	7.34	4.22
v_4	PEP	6.92	9.03	10.06	10.01	14.7	8.43
v'_5	Pyruvate	6.07	7.78	8.58	8.86	12.9	7.11
v_6	Succinate	0.85	1.25	1.47	1.16	1.73	1.32
v_7	Lactate	0.18	0.70	0.22	8.29	11.3	0.14
v_8	Formate	5.89	7.08	6.30	0.56	1.62	5.33
v_9	Hydrogen	3.25	4.58	3.08	0.56	1.62	3.46
v_{10}	Ethanol	2.86	4.23	5.19	0.41	0.97	5.25
v_{11}	Acetate	3.03	2.84	1.11	0.15	0.65	0.08
v_{12}	Acetoin	0.00	0.00	1.03	0.00	0.00	0.82
r(ATP)	ATP	13.41	16.39	16.20	15.17	22.66	12.73

Note. Fluxes (mmol/g-cell h) calculated for $m = n = 12$. G-6-P, glucose 6-phosphate; G-3-P, glyceraldehyde 3-phosphate; PEP, phosphoenol pyruvate.
[a] Biosyn. is the carbon assumed to be converted to biomass from glucose not fermented to fermentation byproducts.

TABLE 5

Ratio of Fluxes Partitioned at the Pyruvate and acetyl-CoA Branch Points

Ratio of flux partitioned to:	acetyl-CoA[a] $\frac{v_8}{v_7+v_{12}}$	lactate[a] $\frac{v_7}{v_8+v_{12}}$	acetate[b] $\frac{v_{11}}{v_{10}}$
GJT001 (laboratory wild type)	32.6	0.03	1.06
GJT001 + pACYC184 (control plasmid)	10.1	0.10	0.67
GJT001 + pAAA215 (ALS plasmid)	5.05	0.03	0.21
YBS125 (*ackA-pta*)	0.07	14.7	—
YBS125 + pACYC184 (control plasmid)	0.14	6.98	—
YBS125 + pAAA215 (ALS plasmid)	5.54	0.02	—

[a] Partitioning at the pyruvate branch point
[b] Partitioning at the acetyl-CoA branch point

carbon flux toward ethanol or acetate production. Competition between the enzymes pyruvate formate lyase (PFL), lactate dehydrogenase (LDH), and ALS show that PFL is the dominant branch. The formation of the ALS enzyme decreases the partition to acetyl-CoA by three- to fivefold.

The effect of the ALS enzyme is not limited to partitioning at the pyruvate branch point. In a strain producing the ALS enzyme, the two- to sixfold decrease in the ratio of fluxes through ACK-PTA and ADH-AD indicates that partitioning at the acetyl-CoA branch point was also affected (Table 5).

Effect of ACK–PTA Mutation

The mutant strain, YBS125, showed an increase in the lactate production rate with a concomitant decrease in formate, hydrogen, ethanol, and acetate production rate when compared with its parent strain, GJT001 (Fig. 2). These observations are consistent with a previous report [12] that showed an increased lactate formation. Excretion of pyruvate was also detected (data not shown). Flux analysis revealed a similar trend in flux redistribution with an approximately 40-fold increased in flux through the lactate branch and 5- to 10-fold decrease in the flux through acetyl-CoA, ethanol, acetate, formate and hydrogen for the YBS125 strain (Table 4).

The slight increase in glucose uptake in the *ackA-pta* mutant is rather unexpected because deficiencies in *ackA* and/or *pta* alone do not effect acetyl-CoA pools [18]. However, since the *ackA-pta* branch enhances acetyl-CoA recycling and promotes growth [19], the observed decrease in cell density of about 30% for the YBS125 strain was not unexpected.

For anaerobic glycolysis, the reduction of acetyl-CoA is believed to be the principle route, for reoxidation of NADH with ethanol as the primary hydrogen acceptor [19, 20]. However, a previous study shows that the elimination of the ADH-AD enzymes leading to the alcohol production pathway did not shuttle NAD regeneration through the lactate branch [19]. Therefore, the observed diversion of flux to lactate is not due solely to the necessity of regenerating NAD.

Acetyl phosphate, the product of catabolism by PTA, acts as a global signal in *E. coli* [21]. Current evidence suggests that a *pta* mutation may induce expression of the fermentative lactate dehydrogenase (LDH) while *ackA* mutation has no apparent effect [22]. Under anaerobic conditions, LDH is induced by low pH and allosterically activated by pyruvate [23–25].

Since no acetyl phosphate is formed from acetyl-CoA in the *pta* mutants, at low pH, acid hydrolysis may result in lower acetyl-phosphate levels. Lowering of the acetyl-phosphate levels alleviates the negative regulation of LDH leading to an overexpression of LDH [22]. An increased intracellular pool of pyruvate coupled with the *pta* mutation may be responsible for a more active LDH and an increased flux to lactate.

The dramatic decrease in the formate, ethanol and hydrogen synthesis rates for the YBS125 strain is unusual since an *ackA-pta* mutation would not be expected to directly affect either the PFL activity or the enzymes in the alcohol production pathway.

Combined Effect of ALS Expression and the ack-pta Mutation

The effect of combining the expression of the ALS enzyme and the elimination of the major acetate production pathway (*ackA-pta*) was unexpected. Except for flux to acetate, the metabolic fluxes and cell density of the combined system is similar to that of the parent strain with the ALS plasmid. A minimal acetate level was obtained for this system. The lactate levels and fluxes decrease dramatically with a concomitant increase in formate and ethanol levels and fluxes. Comparison of the partitioning ratio of the *ackA-pta* mutant with and without ALS plasmid shows that at the pyruvate node, carbon flux can shift readily from lactate to acetyl-CoA (Table 5). The partitioning ratio to the als pathway is fairly constant.

In all the systems considered, the succinate production rate was not affected. Mass conservation calculations showed that the amount of glucose converted to biomass ($_2$) is small except for the *ackA-pta* mutant with the ALS plasmid.

The acetyl-CoA branch point was previously classified as a weakly rigid node with a dominant ethanol and subordinate acetate branch [16]. The decrease in carbon flux to

TABLE 6

K_m Values of *E. coli* Enzymes at the Pyruvate Branch Point

Enzyme	K_m (mM)	References
PFL	2.0	[18, 31]
LDH	7.2	[23]
ALS (*B. subtilis*)	13.0	[32]
PDH	0.3	[33]

ethanol in the *ackA-pta* mutant suggests that removal of this pathway indirectly affects ethanol flux. However, the addition of the ALS plasmid reverts the ethanol flux to levels higher than the wild type. Combining these two observations, our results showed that the *ack-pta* pathway is not the only factor affecting the ethanol synthesis rate.

A comparison of the K_m values at the pyruvate branchpoint shows that the ALS enzyme should not be able to compete effectively with PFL or LDH enzymes. The ability of the ALS enzyme to compete was previously attributed to activation by acetate [7, 33]. However, in the *ackA-pta* mutant the ALS enzyme remains competitive despite a lower level of acetate accumulation. This result indicates that *in vivo* the apparent K_m may be lower than the reported value. Table 6 lists the reported K_m values for the enzymes acting at the pyruvate branch point. The removal of the *ackA-pta* pathway resulted in an increase in lactate production with a concomitant decrease in flux through PFL. Yet, the PFL enzyme is not directly affected since the addition of the ALS plasmid resulted in the recovery of the formate and ethanol production. One probable explanation is that deletion of the *ackA-pta* pathway results in elevated intracellular pyruvate levels which allow LDH to become more competitive versus PFL. Extracellular pyruvate accumulation in *ackA-pta* mutants not carrying the ALS plasmid indicates that this may be the case. The expression of the ALS enzyme redirects the excess pyruvate to acetoin returning the pool to its former levels. The PFL dominates the LDH and returns ethanol and formate production to its former level. This observation suggests the importance of intracellular intermediate concentration in the regulation and redistribution of various metabolic pathways.

CONCLUSION

In this study, we have shown that the host cell existing reaction networks play an important role in the redistribution of metabolic fluxes upon precise genetic perturbations. It is also shown that perturbations in one pathway may have profound effects on other parts of the existing network. This study illustrates the significance of the interplay among various reaction pathways within the network in dictating the final outcome upon any genetic manipulations. Finally, our results also reveals the importance of intracellular intermediate concentration level, in this case pyruvate, in redirecting metabolic fluxes

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