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Effect of a single-gene knockout on the metabolic regulation in *Escherichia coli* for D-lactate production under microaerobic condition

Jiangfeng Zhu[a], Kazuyuki Shimizu[a,b,*]

[a]*Department of Biochemical Engineering & Science, Kyushu Institute of Technology, Iizuka, Fukuoka 820-8502, Japan*
[b]*Institute for Advanced Biosciences, Keio University, Tsuruoka, yamagata, Japan*

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Abstract

The effects of several single-gene knockout mutants (*pyk*F, *ppc*, *pfl*A, *pta*, and *adh*E mutants) on the metabolic flux distribution in *Escherichia coli* were investigated under microaerobic condition. The intracellular metabolite concentrations and enzyme activities were measured, and the metabolic flux distribution was computed to study the metabolic regulation in the cell. The *pfl*A, *pta* and *ppc* mutants produced large amount of lactate when using glucose as a carbon source under microaerobic condition. Comparing the flux distribution and the enzyme activities in the mutants, it was shown that the lactate production was promoted by the inactivation of pyruvate formate lyase and the resulting overexpression of lactate dehydrogenase. The flux through Pta–Ack pathways and the ethanol production were limited by the available acetyl coenzyme A. It was shown that the glycolysis was activated in *pyk*F mutant in microaerobic culture. The glycolytic flux was related with Pyk activity except for *pyk*F mutant. The cell growth rate was shown to be affected by the flux through phosphoenolpyruvate carboxylase. The quantitative regulation analysis was made based on the deviation indexes.

Keywords: *Escherichia coli*; Enzyme activity; Metabolite concentration; Metabolic flux analysis; Lactate production; Pyruvate metabolism

1. Introduction

Under anaerobic or microaerobic condition, *Escherichia coli* converts glucose to a mixture of metabolic products consisting primarily of acetate and formate as well as smaller amounts of lactate, succinate and ethanol (Chatterjee et al., 2001; Clark, 1989). These products have different oxidation states, and by adjusting the proportion of each compound produced, *E. coli* can modulate its metabolism to grow on different carbon sources (Böck and Sawers, 1996; Sawers and Böck, 1988). In *E. coli*, reoxidition of the reducing power produced in the glycolysis mainly through the reaction catalyzed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is made through either lactate production, ethanol production, or succinate production through phosphoenolpyruvate carboxylase (Ppc). Except for succinate, all the fermentation products are formed from pyruvate, and pyruvate plays a key role in the metabolic regulation (Yang et al., 2001). The distribution of the pyruvate pool for organic acid production is under strict control. In *E. coli*, the expressions of the genes encoding the enzymes catalyzing the fermentation and respiration are under the control of some global regulators such as FNR and ArcA/B system. These transcriptional regulators sense the redox state of some intermediates of the respiration process, and repress or activate the genes of the related respiratory or fermentation enzymes. FNR has been known to respond to the

*Corresponding author. Department of Biochemical Engineering & Science, Kyushu Institute of Technology, Iizuka, Fukuoka 820-8502, Japan.
E-mail address: shimi@bse.kyutech.ac.jp (K. Shimizu).

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intracellular [Fe^{2+}/Fe^{3+}] ratio under strictly anaerobic condition, whereas Arc system has more significant effect on the cell growth in microaerobic condition (Drapal and Sawers, 1995; Gunsalus, 1992; Iuchi et al., 1990). Since it has been shown that NADH is a competitive inhibitor of GAPDH in *Lactococcus lactis*, the oxidative state such as NADH/NAD^+ ratio in the cell gives also the important regulation effect on the glycolytic flux (Garrigues et al., 1997).

Energy balance also plays an important role in the metabolic regulation. Under oxygen-limited condition, the respiratory chain is blocked when there are no other electron acceptors. Thus, ATP used for the cell growth is mainly generated through glycolysis and the reaction through acetate kinase (Ack). It has been proved that the flux through glycolysis pathway is controlled by the ATP requirement (Koebmann et al., 2002). Thus the reactions through pyruvate kinase (Pyk) and Ack affect glycolysis for ATP generation. Finally, the flux distribution at the pyruvate node affects the glycolytic flux by changing the related enzyme levels to satisfy both energy and redox balances.

Previous studies have shown that the product spectrum varies by using different substrates with different oxidation states (Alam and Clark, 1989; Böck and Sawers, 1996). Using more oxidized substrates such as glucuronate (oxidation level is 2), the cell produces less lactate and ethanol but more acetate as the end products. On the other hand, if more reduced substrates such as glucitol (oxidation level is −1) were used, the cell produces more lactate. Manipulation of the extracellular oxidoreduction potential changes the intracellular redox state and, therefore, changes the fraction of the metabolic end products (Riondet et al., 2000). It has been shown that the manipulation of the genes related to the pyruvate metabolism significantly changes the product spectrum. In particular, it has been reported that the *pfl* gene knockout (Chang et al., 1999; Zhou et al., 2003; Zhu and Shimizu, 2004) and *pta* gene knockout (Chang et al., 1999) result in the overproduction of lactic acid. It was shown that homo lactate production is achieved under the constraints of both the stoichiometric and the intracellular redox balances in *pfl*A knockout *E. coli* (Zhu and Shimizu, 2004). Metabolic flux analysis gives useful information on the intracellular metabolic regulation. It is considered that the metabolic fluxes represent the final outcome of the cellular regulation at many different levels (Nielsen, 2003). The flux result has to be combined with the enzyme activity result and the intracellular metabolite concentration result to understand the complicated regulation mechanism. The combination of such kinds of information is quite useful for investigation on the effect of the single gene-knockout on the metabolism (Buchholz et al., 2002).

In the present study, several single-gene knockout strains such as *pfl*A, *pta*, *ppc*, *pyk*F, and *adh*E mutants were cultivated using glucose as a carbon source under microaerobic condition. Some intracellular metabolite concentrations and enzyme activities were measured, and the metabolic flux distributions were estimated. The objective of the present research is to investigate the intracellular metabolic regulation in response to the specific gene knockout.

2. Materials and methods

2.1. Organisms and culture conditions

The strains used are the single-gene knockout *E. coli* such as *pfl*A (JW0885), *pta* (JW2294), *ppc* (JW3928), *adh*E (JW1228) and *pyk*F (JW1666) mutants, as well as their parent strain *E. coli* BW25113. The mutants were constructed using the method of Dastenko and Wanner (2000); by Mori et al. (http://www.ttck.keio.ac.jp/IAB/english/research/index.htm). All *E. coli* cells were precultured in a 10 ml L-tube using an LB medium supplemented with 10 g/L of glucose. After 12 h, 1 ml of the culture broth was transferred into 100 ml fresh LB medium in a T-tube. Exponentially growing cells were harvested by centrifuge and used for the inoculation to the main culture. The reactor medium was a minimal medium containing (per liter) 2 g of Na_2SO_4, 2.5 g of $(NH_4)_2SO_4$, 0.5 g of NH_4Cl, 7.3 g of K_2HPO_4, 3.6 g of NaH_2PO_4. The following components were sterilized by passing through a 0.2 μm pore-size filter (per liter of final medium): 3 ml of 1 M $MgSO_4$, and 3 ml trace element solution containing (per liter) 0.5 g of $CaCl_2 \cdot H_2O$, 0.18 g of $ZnSO_4 \cdot 7H_2O$, 0.1 g of $MnSO_4 \cdot H_2O$, 21.1 g of Na_2EDTA, 16.7 g of $FeCl_3 \cdot 6H_2O$, 0.16 g of $CuSO_4 \cdot 5H_2O$ and 0.18 g of $CoCl_2 \cdot 6H_2O$. Glucose was used as a carbon source and the initial concentration was about 10 g/L. Cells were cultivated microaerobically in a 2 liter jar-fermentor (M-100, Rikakikai, Tokyo) with working volume of 1 L at 37 °C. Microaerobic cultures were initiated with 3–8 h of aerobic cultivation followed by the microaerobic cultivation without supplying air and slowing down the agitation speed to around 50 rpm so that the dissolved oxygen concentration (DO) decreased to less than 5% of air saturation. The pH was maintained at 7.0 by automatic addition of 4 M of NaOH or 2 M of HCl.

2.2. Analyses

Cell dry weight was determined by measuring the optical density at 600 nm (OD_{600}). Samples were centrifuged for 10 min at 4 °C and 6000 × *g* to remove the cells for extracellular metabolite analysis. The glucose concentration was measured by a colorimetric

method using a kit (Wako Pure Chemical Co., Osaka, Japan). The concentrations of D-lactate, acetate, formate and succinate were measured using capillary electrophoresis (CE) (Agilent, Germany). Ethanol concentration was measured using a kit (Boehringer Mannheim, Germany). Oxygen and carbon dioxide concentrations in the off gas were determined using a gas analyzer (DEX-2562, Able Co., Tokyo, Japan).

2.3. Determination of intracellular metabolite concentrations

About 10 ml of the culture broth was quickly taken as the sample, and immediately injected into 40 ml of pre-cooled 60% methanol. The mixture was kept at −20 °C. The quenched samples were centrifuged at 10,000 × *g* at a temperature of −15 °C for 10 min. The pellets were kept at −20 °C for 30 min and then lyophilized. The dried biomass was kept in a −20 °C freezer for further processing. Intracellular metabolites except NADH and NADPH were extracted by perchloric acid in ethanol. The extract was placed on ice-bath for 10 min and then neutralized with K_2CO_3. Then, the cell debris and $KClO_4$ were removed by centrifugation (Williamson and Corkey, 1969).

For NADH measurement, alkaline extraction was utilized, and acid extraction method was used for other metabolite measurements. Nucleotides concentrations were measured according to Williamson and Corkey (1969). The concentrations of glucose 6-phosphate (G6P), fructose 1,6-bisphosphate (FDP), phosphoenolpyruvate (PEP) and pyruvate (PYR) were measured according to Schaefer et al. (1999). The concentration of acetyl coenzyme A (AcCoA) was measured following to Riondet et al. (2000), and ATP, ADP, AMP as well as NAD^+ were measured based on Bergmeyer's protocol (1984, 1985).

2.4. Enzyme assays

The culture broth with a volume corresponding to about 100 mg (dry weight) of cells was centrifuged (4 °C, 10 min at 10,000 × *g*) and washed twice with 20 mM Tris-HCl buffer (pH at 7.0). Cells were resuspended in Tris (100 mM) -HCl buffer (pH at 7.0) containing KCl (20 mM), $MnSO_4$ (5 mM), DTT (2 mM) and EDTA (0.1 mM). Cell disruption by sonication (five cycles of 30 s with 1 min cooling periods) was followed by the removal of cell debris by centrifugation for 10 min at 10,000 × *g* at 4 °C. The supernatant was used for all enzyme assays. The protein concentration of extracts was determined by the Lowry's method (1951) with bovine serum albumin used as the standard.

Enzyme assays were made based on the relationships between the enzyme activity and the consumption or production of NADH or NADPH monitored at 340 nm in a spectrophotometer. The enzyme activities of glucose 6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) were measured based on the methods of Moritz et al. (2000). The enzyme activities of Pyk, lactate dehydrogenase (LDH), Ack, Ppc and alcohol dehydrogenase (ADH) were measured by Riondet et al.'s method (2000). The enzyme activity of GAPDH was measured based on the method of Even (1999). The enzyme activity of pyruvate-formate-lyase (Pfl) was measured based on Garrigues et al. (1997).

3. Results

Several single gene-knockout mutants as well as their parent strain *E. coli* BW25113 were cultivated under microaerobic condition using glucose as a sole carbon source. The fermentation data are summarized in Table 1. The parent strain BW25113 converted most of the glucose to biomass and organic acids such as formate and acetate. The yield of ethanol was comparable to that of acetate. Succinate, lactate and pyruvate were the minor products for this strain, where the yields of these products were less than 5%. The parent strain showed a typical mixed acid fermentation under microaerobic condition. It has been reported that the complete knockout of *ppc* gene may not allow *E. coli* to

Table 1
Comparison of the fermentation results

Strain	Specific growth rate μ (h^{-1})*	Yield on glucose (g/g)%*						
		Biomass	Lactate	Acetate	Formate	Succinate	Ethanol	Pyruvate
BW25113	0.85±0.08	30.1±0.	1.2±0.0	24.3±0.1	30.0±0.2	5.0±0.2	14.7±0.3	0.2±0.0
*Pfl*A$^-$	0.18±0.04	8.1±0.1	72.5±0.0	1.1±0.1	0.0±0.2	0.0±0.0	1.0±0.0	1.1±0.0
pta$^-$	0.24±0.04	6.9±0.1	51.3±0.0	1.6±0.1	2.1±0.2	4.5±0.1	0.0±0.0	0.0±0.0
ppc$^-$	0.01±0.02	4.6±0.1	32.2±0.0	13.5±0.1	9.4±0.2	0.3±0.0	6.2±0.1	4.6±0.0
*adh*E$^-$	0.03±0.02	11.4±0.1	9.7±0.0	42.4±0.1	12.1±0.2	3.2±0.1	0.2±0.0	0.0±0.0
*pyk*F$^-$	0.48±0.05	13.0±0.1	2.1±0.0	34.0±0.1	22.9±0.2	6.9±0.2	12.0±0.2	0.0±0.0

*The data were calculated based on the cultivation result during the exponential phase.

Table 2
Comparison of some related enzyme activities

	Enzyme activities (mmol/min mg protein)*								
	G6PDH	6PGDH	GAPDH	Pyk	LDH	Ppc	Pfl	Ack	ADH
BW25113	0.152±0.023	0.18±0.002	0.008±0.001	0.657±0.102	0.503±0.120	0.008±0.003	0.071±0.012	0.038±0.006	0.006±0.000
*pfl*A$^-$	0.252±0.061	0.079±0.010	0.076±0.011	0.726±0.170	2.093±0.512	0.343±0.122	0.004±0.004	4.088±0.200	0.011±0.002
pta$^-$	0.187±0.027	0.001±0.001	0.060±0.008	0.512±0.006	1.621±0.306	0.005±0.001	0.013±0.001	3.465± 0.195	0.005±0.001
ppc$^-$	0.017±0.002	0.000±0.000	0.014±0.001	0.183±0.060	0.164±0.020	0.003±0.002	0.025±0.006	0.629±0.035	0.040±0.009
*adh*E$^-$	0.013±0.002	0.004±0.001	0.008±0.003	0.108±0.021	0.000±0.000	0.005±0.001	0.043±0.001	0.536±0.035	0.000±0.000
*pyk*F$^-$	0.052±0.006	0.017±0.003	0.020±0.005	0.009±0.003	0.069±0.030	0.013±0.001	0.050±0.004	0.293±0.016	0.001±0.001

*All the enzyme activities are the average of at least three measurements.

grow in a minimal media using glucose as a sole carbon source (McAlister et al., 1981). In the present study, we used LB medium for the preculture. This may have affected the recovery of ppc gene. The enzyme activity of Ppc was present in this strain although very low level (Table 2). Therefore, the present study shows the effect of significantly reducing the Ppc activity on the metabolism.

Table 1 shows that the metabolic modes were significantly changed by the single-gene knockouts. The biomass synthesis was significantly defected in the mutant strains. A significant lactate production was observed in *ppc*, *pflA*, and *pta* mutants, in which the lactate yields on glucose varied from 32.2% to 72.5%. The subsequent products of the reaction catalyzed by Pfl such as acetate, formate and ethanol were almost negligible in *pfl*A and *pta* mutants. The yields of these metabolites in *ppc* mutant were less than half of that in the parent strain. Ethanol production was comparable to that of acetate in all the strains except for *adh*E mutant, in which the acetate yield was more than 200 times higher than that of ethanol. This result explicitly indicates that the partition of the flux at AcCoA node was forced to acetate production by *adh*E gene-knockout. The acetate yield in *pyk*F mutant was also relatively higher than the parent strain as well as other gene knockout strains such as *pfl*A, *pta*, and *ppc* mutants. As compared with other mutants, significant amount of succinate and ethanol were found in the culture broth of *pyk*F mutant.

3.1. Effect of a single-gene knockout on enzyme activities

Many of the metabolic adjustments in response to a specific gene knockout, and/or the changes in culture conditions are accompanied by the changes in metabolite concentrations and enzyme activities. Some of the enzyme activities were measured in the present study to investigate the protein expression levels (Table 2). The *pfl*A mutant showed much higher activities of GAPDH (about 9 times higher), LDH (about 3 times higher), Ppc (about 100 times higher) and Ack (about 100 times higher) as compared with the parent strain BW25113. The up-regulation of GAPDH and LDH implies the coupling between the NADH production and consumption between the two corresponding reactions. The specific Pyk activity of *pfl*A mutant was higher than that of the parent strain BW25113. Since ATP can only be produced through substrate level phosphorylations in the glycolysis and Pta–Ack pathway, the up-regulation of the enzyme activities of Pyk and Ack implies ATP requirement for the cell. Similar to *pfl*A mutant, the simultaneous up-regulation of GAPDH (more than 7 times) and LDH (about 3 times) were also observed in the *pta* mutant (Table 2). Interestingly, Pfl was inactivated in this mutant, which implies a common regulatory mechanism that controls the expression of *pta* and *pfl* genes. The significant difference between *pta* and *pfl*A mutants was the specific activity of Ppc. In *pta* mutant, this enzyme activity was not much changed as compared with the parent strain BW25113. However, *pta* mutant produced more succinate than *pfl*A mutant despite of the lower Ppc activity (Table 1), which implies that the intracellular pool size of PEP played another important role in the metabolic regulation.

The enzyme activities of Ack and ADH, both of which are the AcCoA assimilation pathway enzymes, increased in *ppc* mutant. However, the enzyme activity of Pfl, which supplies AcCoA for these two reactions, was much lower than that of the parent strain BW25113 (Table 2). Less acetate and ethanol were produced in *ppc* mutant than those of the parent strain. Although the activity of LDH was lower, lactate production was higher in *ppc* mutant as compared with that of the parent strain BW25113. The activity of Pyk in *ppc* mutant was about $\frac{1}{4}$ of that in the parent strain. Pyk activity in *adh*E mutant was also found to be down regulated. The activity of Ack was higher in these two strains and *pyk*F mutant, which may be due to ATP generation under oxygen limited condition. The activities of LDH and Pfl were both down regulated in *adh*E mutant, which is consistent with the relatively lower lactate and formate yield in this strain (Table 1). The *pyk*F mutant showed higher Ppc activity while the

activities of LDH and ADH were both down regulated as compared with the parent strain.

3.2. Effect of a single-gene knockout on intracellular metabolite concentrations

To investigate the intracellular metabolism, several intracellular metabolite concentrations were also measured, and the results are shown in Table 3. The pool sizes of G6P, FDP, and PYR increased 3.5 to 20 times in *pfl*A mutant as compared with the parent strain. The PEP concentration was lower in *pfl*A mutant. Since PEP is involved in the phosphotransferase system (PTS) in *E. coli*, the lower PEP concentration is consistent with the higher glucose uptake rate and the higher Pyk activity in this mutant. The low PEP concentration causes less succinate production even with high Ppc activity. The metabolite concentrations in *pta* mutant were similar to those in *pfl*A mutant except for significantly accumulated PEP. Glycolytic metabolites were accumulated in the mutants such as *pfl*A, *pta*, *ppc*, and *adh*E mutants. In the case of *pyk*F mutant, all intracellular metabolites in glycolysis were significantly accumulated as compared with the parent strain and other mutants. Two of the lactate producing strains such as *pfl*A and *pta* mutants showed higher NADH/NAD^+ ratio as compared with the parent strain. Therefore, it is reasonable to consider that the lactate production was promoted by the intracellular redox balance pressure in these two strains. AcCoA was not accumulated in the cells despite of the gene-knockouts such as *adh*E and *pta* mutation. The value of ATP /AXP (ATP + ADP + AMP) was less in the mutants as compared with the parent strain, and this ratio was low in *pta* and *pyk*F mutants.

3.3. Effect of a single-gene knockout on the flux distribution

The intracellular metabolic flux distribution was estimated for each strain using the fermentation data during the exponential growth phase. The flux profiles were estimated from metabolite balances as described by Vallino and Stephanopulos (1993). Based on the biochemistry framework as shown in Fig. 1, the constraints imposed by the stoichiometry and the measured specific rates during the exponential phase were used to obtain the flux distribution.

The flux values were shown in Table 4. Although one of the catabolic pathways is blocked by gene knockout, the higher glucose uptake rate (v1), followed by higher glycolytic flux, was found in the strains such as *pfl*A, *pta*, and *pyk*F mutants as compared with the parent strain. In particular, the glucose uptake rate was about 40% higher in *pyk*F mutant than that in *E. coli* BW25113. This is consistent with the significantly higher intracellular concentration levels of the glycolytic metabolites in

Table 3
Comparison of the intracellular metabolite concentrations in the *E. coli* mutants

	Metabolite concentration (mM/g DCW)*											
	G6P	FDP	PEP	PYR	AcCoA	ATP	ADP	AMP	ATP/AXP	NADH	NAD	NADH/NAD
BW25113	0.05 ± 0.01	4.59 ± 0.02	0.32 ± 0.12	8.21 ± 0.01	0.07 ± 0.00	3.06 ± 0.17	0.38 ± 0.01	0.21 ± 0.01	0.84	0.018 ± 0.002	0.143 ± 0.001	0.126
*pfl*A$^-$	1.09 ± 0.01	16.41 ± 0.11	0.12 ± 0.04	25.49 ± 0.01	0.05 ± 0.00	1.69 ± 0.11	0.20 ± 0.01	0.20 ± 0.01	0.81	0.056 ± 0.001	0.060 ± 0.002	0.933
pta$^-$	0.54 ± 0.03	15.08 ± 0.03	1.27 ± 0.20	21.83 ± 0.55	0.01 ± 0.00	1.82 ± 0.16	1.17 ± 0.08	1.12 ± 0.17	0.44	0.047 ± 0.001	0.129 ± 0.007	0.680
ppc$^-$	0.84 ± 0.03	24.46 ± 0.04	0.96 ± 0.07	38.72 ± 2.26	0.05 ± 0.02	4.19 ± 0.07	3.71 ± 1.14	1.16 ± 0.43	0.53	0.018 ± 0.004	0.170 ± 0.010	0.106
*adh*E$^-$	0.32 ± 0.01	11.41 ± 0.03	0.46 ± 0.08	18.40 ± 0.25	0.01 ± 0.00	1.38 ± 0.04	0.69 ± 0.02	0.46 ± 0.04	0.55	0.010 ± 0.006	0.058 ± 0.009	0.167
*pyk*F$^-$	1.15 ± 0.02	30.90 ± 0.90	3.67 ± 0.06	46.96 ± 1.98	0.08 ± 0.02	2.65 ± 0.10	2.27 ± 0.05	1.01 ± 0.08	0.47	0.002 ± 0.005	0.177 ± 0.008	0.010

*The deviations are given by at least three measurements.

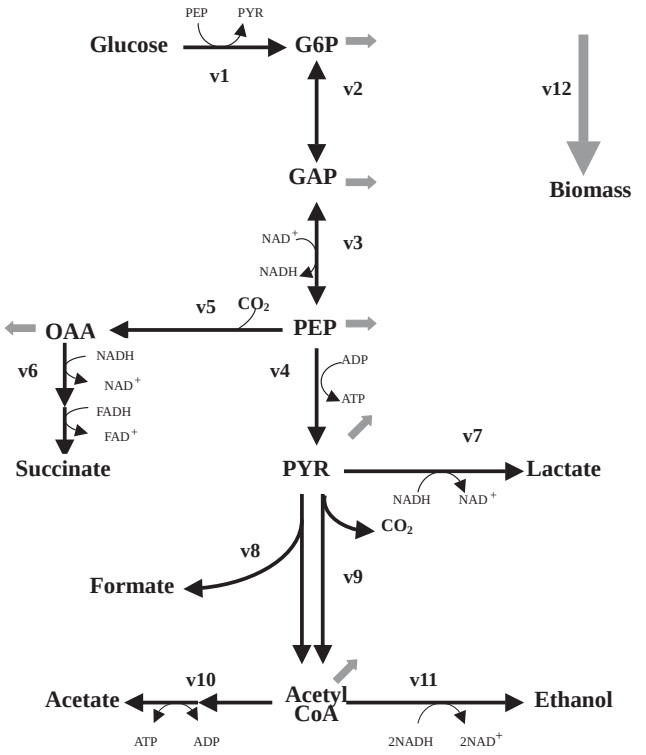

Fig. 1. General metabolic pathway of *E. coli* under oxygen-limited condition. The fluxes through each pathway are designated v1–v12. Abbreviations: G6P, glucose 6-phosphate; GAP, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate; OAA, oxaloacetate; PYR, pyruvate.

*pyk*F mutant. The flux through GAPDH (v3) was shown to be regulated by the intracellular NADH/NAD^+ ratio (De Graef et al., 1999; Garrigues et al., 1997). It has been shown that NADH competitively combines to GAPDH and inhibits the reaction through this enzyme. The GAPDH activity in the mutant strains such as *pfl*A, *pta*, and *pyk*F mutants were 9.5-fold, 7.5-fold, and 2.5-fold of that in the parent strain, respectively (Table 2). The intracellular NADH/NAD^+ ratio was significantly lower in *pyk*F mutant (Table 3), and, therefore, the higher glycolysis flux was not restricted by the competitive inhibition of NADH on the reaction through relatively high level of GAPDH. While the intracellular NADH/NAD^+ ratio was significantly higher in *pfl*A and *pta* mutants than the other strains. Lactate was produced in these two mutants to regenerate NAD^+ needed for glycolysis. Correspondingly, the GAPDH expression level was also significantly high in these two strains. These results indicate that the demand for glycolytic flux dominates over the intracellular redox balance. The high glycolytic flux resulted in high intracellular NADH/NAD^+ ratio, and subsequently lactate was produced to regenerate NAD^+ so that the glycolysis continues to work.

Table 4
Effect of a single-gene knockout on the flux distribution

	Fluxes (mmol/g DCW/h)*											
	v1	v2	v3	v4	v5	v6	v7	v8	v9	v10	v11	v12
BW25113	6.30±0.06	5.90±0.07	11.73±0.10	3.85±0.09	1.39±0.09	0.42±0.06	0.09±0.06	7.28±0.35	1.97±0.23	4.47±0.12	3.52±0.11	3.94±0.10
*Pfl*A$^-$	8.15±0.06	7.58±0.07	14.93±0.10	5.71±0.10	0.78±0.10	0.18±0.11	11.69±0.7	0.00±0.00	1.64±0.11	0.46±0.2	0.45±0.10	1.62±0.11
Pta$^-$	6.51±0.06	5.79±0.06	11.27±0.10	3.03±0.10	1.37±0.10	0.77±0.10	7.36±0.83	0.70±0.06	0.92±0.10	0.61±0.3	0.31±0.12	1.26±0.12
Ppc$^-$	1.59±0.02	1.48±0.02	2.90±0.03	1.12±0.02	0.14±0.02	0.00±0.03	1.13±0.1	0.62±0.07	0.66±0.03	0.72±0.08	0.45±0.06	0.20±0.02
AdhE$^-$	1.10±0.02	1.04±0.02	2.05±0.03	0.76±0.04	0.15±0.03	0.07±0.02	0.23±0.03	0.53±0.06	1.03±0.05	1.45±0.05	0.00±0.02	0.27±0.04
*pyk*F$^-$	9.02±0.07	7.05±0.07	13.21±0.07	0.00±0.00	3.21±0.33	1.54±0.23	0.13±0.2	7.82±0.29	2.56±0.43	7.75±0.4	2.93±0.31	2.48±0.23

*The errors were derived from the detection errors.

Table 5
Effect of a single-gene knockout on flux partitions

	Percentage partitioned at PEP node to pyruvate $\left(\frac{v_1+v_4}{v_1+v_4+v_4}\times 100\right)$	Percentage partitioned at pyruvate node to lactate $\left(\frac{v_7}{v_7+v_8+v_9}\times 100\right)$	Percentage partitioned at AcCoA node to acetate $\left(\frac{v_{10}}{v_{10}+v_{11}}\times 100\right)$
BW25113	88.0	1.0	55.9
*pfl*A$^-$	94.7	87.7	50.5
pta$^-$	87.4	82.0	66.3
ppc$^-$	95.1	46.9	61.5
*adh*E$^-$	92.5	12.8	100.0
*pyk*F$^-$	73.8	0.0	72.6

The lactate producing flux (v7) in *ppc* mutant was lower than those of the other two lactate producing mutants such as *pfl*A and *pta* mutants. It is consistent with the lower glycolytic flux in *ppc* mutant. As compared with the parent strain, the lactate production rate in *ppc* mutant was about 7 times higher. However, the lactate yield was about 30-fold higher as compared with that of the parent strain (Table 1). Comparing the glucose uptake rates (v1) in these two strains, the glycolytic flux in *ppc* mutant was about 17% of that in *E. coli* BW25113. It was shown from Table 5 that the percentage of the flux partitioned at pyruvate node to lactate was significantly higher in *ppc* mutant as compared with the parent strain. The ratio was even higher in the other lactate producing strains such as *pfl*A and *pta* mutants. It is known that the reactions through LDH and Pfl are competing with each other (Böck and Sawers, 1996). The high intracellular NADH/NAD$^+$ ratio is a plausible reason of the significantly up-regulated LDH activity and down-regulated Pfl activity in *pflA* and *pta* mutants (Tables 2 and 3).

The flux through Ppc (v5) in *pyk*F mutant was about 2.6-fold of that in the parent strain. From the enzyme activity data, it can be seen that the Ppc activity in *pyk*F mutant was up-regulated for about 60% as compared with the parent strain. The reason for the high Ppc flux may be due to the high substrate (PEP) concentration and the synergistic activation by FDP (Table 3) (Smith et al., 1980; McAlister et al., 1981). The Ppc flux was low in *ppc* and *adh*E mutants as compared with the parent strain, which is consistent with the significant high relative flux going toward pyruvate at PEP node (Table 5). Small residual Ppc activity was found in *ppc* mutant by enzyme activity measurement. Since the product of the reaction through Ppc is the precursor of some amino acids such as aspartate and asparagine, the low Ppc flux is consistent with the low biomass synthesis flux (v12) in these two mutants. As a consequence, the glycolytic flux, which is used to supply energy for cell growth, was low in *ppc* and *adh*E mutants.

It can be seen from Table 5 that the flux partitioning at AcCoA node was significantly different in *adh*E and *pyk*F mutants as compared with the others. The reaction through ADH to ethanol was blocked by *adh*E gene knockout, and therefore AcCoA can only be used for cell growth and acetate production. While in *pyk*F mutant, the flux distribution at AcCoA node was determined by the expression of Ack and ADH (Table 2). The Ack expression in *pfl*A and *pta* mutants was significantly up regulated. However, the ratios of the flux partitioned to acetate were 50.5% and 66.3% in *pfl*A and *pta* mutants, respectively, which are not much changed by the high Ack activities in these two strains as compared with the ratio of 55.9% in the parent strain.

4. Discussion

4.1. Flux partitioning at the pyruvate node

Pyruvate is competed by the reactions through Pfl and LDH. In the wild type *E. coli*, LDH reaction is not as competitive as the reaction through Pfl, and therefore, acetate and formate were the main metabolites instead of lactate (Kessler and Knappe, 1996; Kessler et al., 1992). However, the knockouts of *pfl*A, *pta* and *ppc* genes significantly changed the enzyme expression and intracellular states, resulting in the overproduction of lactate. The knockout of *pfl*A gene blocked the pyruvate assimilation through Pta–Ack and ADH pathways, which are usually used for ATP production and NADH reoxidition, respectively, in the wild-type *E. coli*. Since the glycolytic flux is promoted by anaerobiosis with higher ATP requirement, lactate was produced to satisfy both the stoichiometric and intracellular redox balances (Zhu and Shimizu, 2004). Looking at the enzyme activity results (Table 2), the LDH activity was up-regulated by about 3-fold as compared with the parent strain. Similar phenomena were found in *pta* mutant. The Pfl activity in *pta* mutant was about one-fifth of that in the parent strain, and LDH activity was up-regulated by more than two folds. As a result, the flux through Pfl (v8) was about one tenth of that in the parent strain BW25113 (Table 4), and the ratio of flux partitioned at

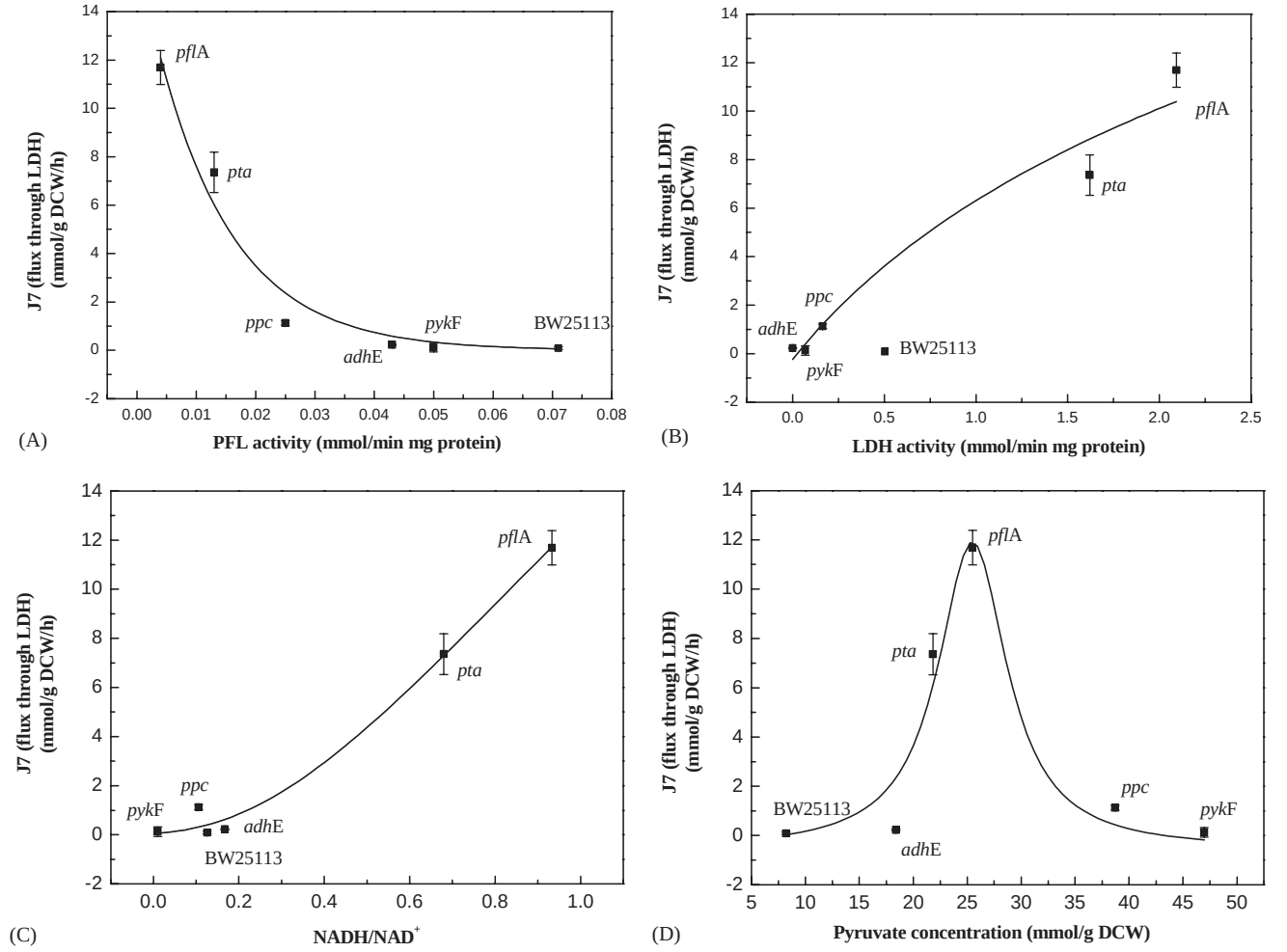

Fig. 2. Influence factors of lactate producing flux. The effects of Pfl, LDH activities, NADH/NAD$^+$ ratio, and intracellular pyruvate concentration on flux toward lactate generation are represented in A, B, C, and D, respectively. Error bars indicate deviation of fluxes.

pyruvate node to lactate was comparable to that in *pfl*A mutant (Table 5).

The competition for PYR by the reactions through Pfl and LDH is shown in Fig. 2 by plotting the LDH fluxes against Pfl and LDH activities in different strains. The LDH flux increased as the Pfl activity became low. The change in LDH activity also contributed to the LDH flux. The deviation index of the specific flux J with respect to the enzyme E, defined as $(\Delta J/\Delta E)(E^r/J^r)$ (Small and Kacser, 1993) was computed to compare the effects of LDH and Pfl activities on the lactate production. The deviation index is related to the control coefficient, and can be used as a measure of the effects of large changes in enzyme activities or other effectors on the flux (Yang and San, 1999; Stephanopoulos et al., 1998). The superscript r refers to the reference value at the new perturbed state. Since the enzyme activity varied "largely" to the wild type state, the deviation indexes were evaluated using the neighboring points as the original state (Table 6). Table 6 shows that LDH controls the lactate production in *pfl*A mutant where the deviation index for LDH flux with respect to Pfl was low due to the low Pfl activity value. Pfl significantly dominates over LDH in the cases of *ppc* and *adh*E mutants. This maybe explained why there was still high lactate production in *ppc* and *adh*E mutants although the LDH activity was lower as compared with the parent strain. The large value of the deviation index of *adh*E mutant for LDH flux with respect to Pfl was due to the

Table 6
Deviation index for LDH flux

New state	Original state	Deviation index for LDH flux	
		$\left(\frac{\Delta J_7}{\Delta LDH}\right)\left(\frac{LDH^r}{J_7^r}\right)$	$\left(\frac{\Delta J_7}{\Delta PFL}\right)\left(\frac{PFL^r}{J_7^r}\right)$
*pfl*A$^-$	*pta*$^-$	1.64	−0.16
pta$^-$	*ppc*$^-$	0.94	−0.92
ppc$^-$	adhE	0.80	−1.11
*adh*E$^-$	*ppc*$^-$	0.00	−9.35
*pyk*F$^-$	BW25113	−0.05	−0.73

low LDH flux value in this mutant. This result is in contrast with the previous work on *Lactococcus lactis* (Andersen et al., 2001), which showed that the control by lactate dehydrogenase on lactate production was close to zero. In their study, the LDH activities ranged from 1% to 133% of that in the wild type. In the present work, similar result was obtained when the LDH activity was comparable or lower than that in the wild type. However, further amplification of LDH activity to 3–5-folds higher by corresponding gene modification showed that the amplified LDH regained control on the LDH flux. Similar result was also obtained by Yang and San (1999), who showed that the overexpression of LDH from 1.3 to 15.3 units significantly increased lactate production and the deviation index was about 0.57 and 1.16, respectively according to different original states. In the present work, the simultaneous decrease in the activity of Pfl also contributed to the increase in LDH flux. Table 6 shows that the LDH and Pfl activities have approximately equal control on the lactate production in *pta* mutant.

Looking at the relationship between the flux through LDH (v7) and the intracellular NADH/NAD$^+$ ratio (Fig. 2C), it is clear that the high flux toward lactate production or the high LDH activity might be induced by the high intracellular redox balance pressure. Similar to the deviation index, the sensitivity index, $(\Delta J/\Delta P)(P^r/J^r)$, was defined (Small and Kacser, 1993; Yang et al., 2001), where ΔP is the perturbation of the intracellular metabolite concentration. In *pfl*A and *pta* mutants, the sensitivity indexes for LDH flux with respect to NADH/NAD$^+$ ratio were 1.37 and 1.28, respectively, which indicates that the higher intracellular redox balance pressure causes more lactate formation. The effect of intracellular pyruvate concentration on LDH flux (v7) was shown in Fig. 2D. Although the flux toward lactate production increased as the pyruvate concentration increases, further increase in pyruvate concentration reduced the LDH flux in *ppc* and *pyk*F mutants. The pyruvate is the substrate for LDH reaction. However, the high lactate production in *pfl*A and *pta* mutants cannot be simply explained by the high intracellular pyruvate concentration. The results indicated that the high LDH activities and NADH/NAD$^+$ ratios contributed more than the intracellular pyruvate to the high LDH flux in these two strains. It should be noted that the high pyruvate concentration in the mutants were the result of several factors such as glucose uptake rate, Pfl and LDH activities, etc.

4.2. Flux partition at the AcCoA node

In the parent strain and its several mutants such as *pfl*A, *pta*, and *ppc* knockout strains, the percentage of the flux partitioned at AcCoA node to acetate showed a small change, which varied from 50.5% to 66.3% (Table 5) (AcCoA used for biomass synthesis was not considered to calculate the flux partition at AcCoA node). Studies of the strictly anaerobic cultivation have shown that the ratio of acetate production and ethanol production is about 1:1 in the wild-type *E. coli* (Alexeeva et al., 2000; Vemuri et al., 2002). This ratio may be affected by the available AcCoA and the intracellular redox balance caused by the residual activity of PDH (Alexeeva et al., 2000; De Graef et al., 1999). The flux partition at the AcCoA node was different in *pyk*F and *adh*E mutants (Table 5). Most AcCoA was forced to Pta–Ack pathway to form acetate in *adh*E mutant, since the ethanol formation pathway is blocked. However, the higher acetate production in *pyk*F mutant as compared with the parent strain was unexpected. Previous research has shown that the higher intracellular pyruvate concentration favors acetate production (Yang et al. 2001). However, this may not be the case in the present work. It was found that no AcCoA accumulation occurred while it was expected in the mutants such as *pta* and *adh*E strains. The relationship between the Pta–Ack flux and the intracellular AcCoA concentration can be seen in Fig. 3A. The sensitivity indexes for Pta–Ack flux with respect to AcCoA concentration in *pyk*F mutant and the parent strain were 0.93 and 0.79, respectively. It indicates that the AcCoA availability is a limiting factor for relatively high flux toward acetate formation. Fig. 3C shows that the Pta–Ack flux increases as Pfl activity increase. The control of Pfl on Pta–Ack flux was not significant in *pfl*A and *pta* mutants which showed low acetate production. The deviation indexes for Pta–Ack flux with respect to Pfl activity was 0.15 and 0.36 for *pfl*A and *pta* mutants respectively. However, it was shown that the LDH flux was significantly controlled by LDH in these two strains. Therefore, the flux through Pfl, the competitive process of LDH, was low due to the high LDH activity in *pfl*A and *pta* mutants. Therefore, the Pta–Ack flux depends on the flux through Pfl for AcCoA supply.

4.3. Regulation of glycolysis and flux partitioning at the PEP node

The reaction from PEP to oxaloacetate (OAA) through Ppc is the main pathway to replenish oxaloacetate (OAA) in *E. coli* under oxygen-limited condition (Vemuri et al., 2002). Succinate is derived from OAA, and two equivalents of NADH are required for one mole of succinate production. In PTS system, PEP is used to transport phosphate for glucose utilization. The reaction through Pyk also uses PEP to produce PYR and ATP.

Previous studies have reported that the glycolysis was down regulated in *E. coli pyk*F mutant under aerobic condition (Emmerling et al., 2002; Siddiquee et al., 2004). However, when *pyk*F mutant was cultivated

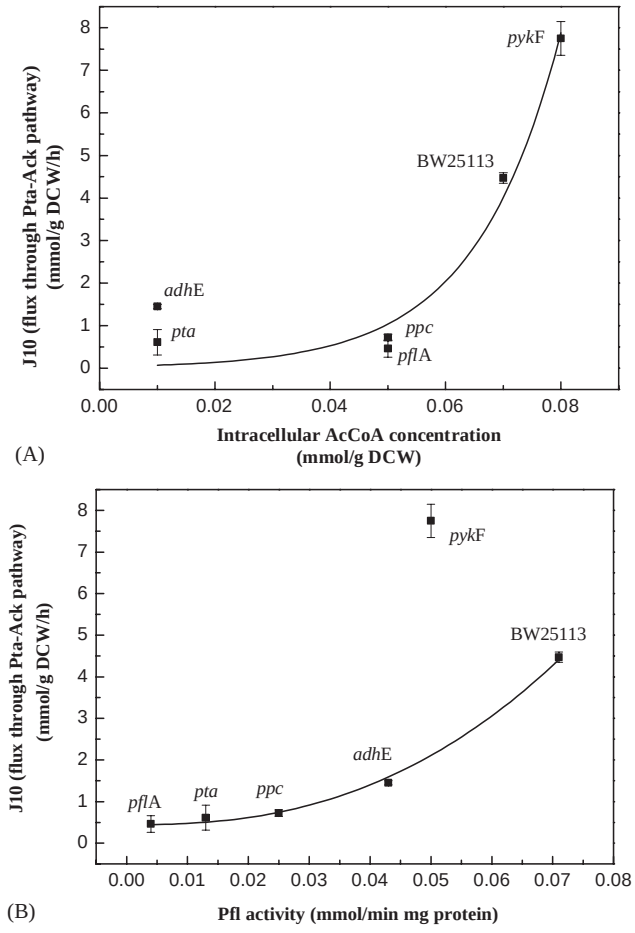

Fig. 3. Influence factors of flux through Pta–Ack pathway. The effects of intracellular AcCoA concentration and Pfl activity are shown in A and B, respectively. Error bars indicate deviation of fluxes.

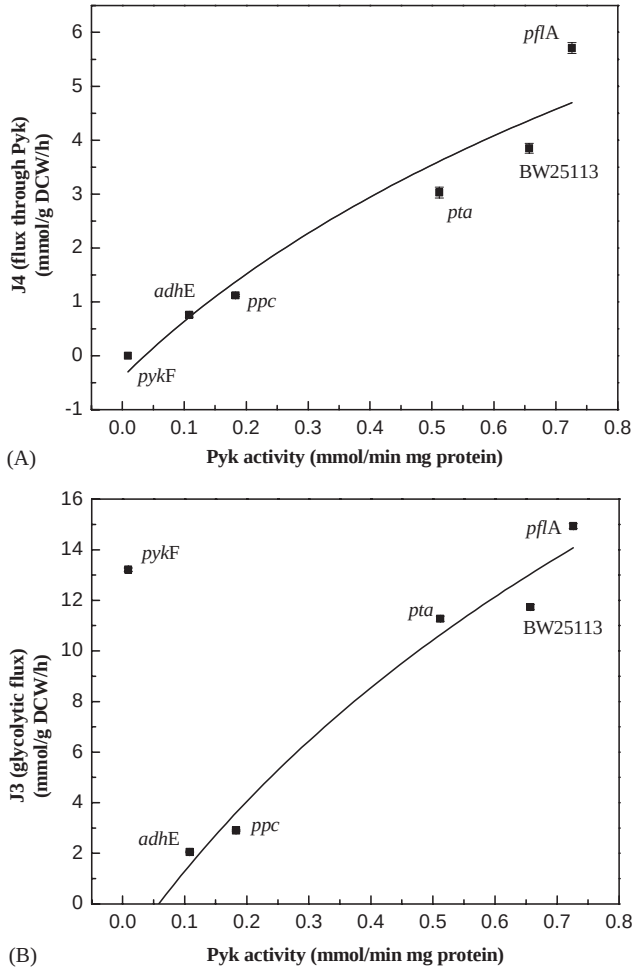

Fig. 4. The effect of Pyk activity on (A) Pyk flux and (B) glycolytic flux. Error bars indicate deviation of fluxes.

under microaerobic condition, the specific glucose uptake rate increased about 50% as compared with the parent strain. Flux through glycolysis was reported to be controlled by the ATP requirement in *E. coli*. By optimizing additional ATP hydrolysis, the glycolytic flux was increased significantly (Koebmann et al., 2002). Under oxygen-limited condition, ATP is considered to be produced by the reactions through Pyk and Ack pathway due to ATP balance. It was shown that the flux through Ack reaction is controlled by the flux through Pfl instead of Ack activity. The changes in the glycolytic flux and the flux through Pyk with respect to Pyk activity are shown in Fig. 4. The deviation indexes for Pyk flux with respect to Pyk activity were calculated to be around 1 for all the experimental strains which indicates that this flux is controlled by Pyk enzyme. Pyk enzyme also showed significant control on the glycolytic flux (Fig. 4B). The deviation indexes for glycolytic flux (v3) with respect to Pyk activity change varied from 0.60 to 1.16 in the strains used except for *pyk*F mutant, in which the glycolytic flux was obviously independent of Pyk activity. The regulation of PTS system was most probably exerted by the global transcriptional control factors such as cAMP–CRP complex and the catabolite repressor/activator protein (Cra) (for reviews, see Saier and Ramseier, 1996; Titgemeyer and Hillen, 2002). The pohosphoriated IIA^{Glc} level is related to the energy status in the cell and it controls the activity of adenylate cyclase. Therefore, IIA^{Glc} controls the cAMP level according to the cell status. cAMP–CRP complex is estimated to control more than 100 genes in *E. coli*, including *pts*H and *crr* genes (Titgemeyer and Hillen, 2002; Reuse and Danchin, 1988). In aerobic cultures of *E. coli*, ATP is mainly provided by the oxidative phosphorylation. Lacking of the reaction through Pyk does not significantly affect the ATP level in vivo. Therefore, the reduced glycolytic flux in the aerobically cultivated *pyk*F mutant follows different regulation.

Since oxaloacetate is one of the important precursors for the cell synthesis, the flux through Ppc was considered to affect the cell growth, which can be seen in Fig. 5. It indicates that the single gene knockouts

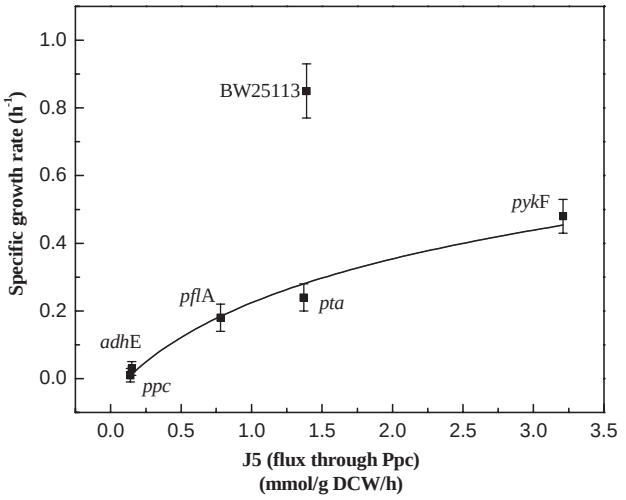

Fig. 5. Effect of Ppc flux on cell growth rate. Error bars indicate deviation of fluxes.

significantly reduced the growth efficiency. This can be also found by the low biomass yields in the mutants (Table 1). The Ppc activity in *pfl*A was very high as compared with other strains (Table 2). It may be considered to be due to intracellular redox balance pressure. Although the Ppc activity was high, the intracellular PEP concentration was very low in *pfl*A mutant as compared with other strains. Therefore, the flux through Ppc (v5) was relatively low in this mutant.

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