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ORIGINAL PAPER

Effect of *poxB* gene knockout on metabolism in *Escherichia coli* based on growth characteristics and enzyme activities

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Abstract The effect of *poxB* gene knockout on metabolism in *Escherichia coli* was investigated in the present paper based on the growth characteristics and the activities of the enzymes involved in the central metabolic pathways. The absence of pyruvate oxidase reduced the glucose uptake rate and cell growth rate, and increased O_2 consumption and CO_2 evolution. The enzyme assay results showed that although glucokinase activity increased, the flux through glycolysis was reduced due to the down-regulation of the other glycolytic enzymes such as 6-phosphofructosekinase and fructose bisphosphate aldolase in the *poxB* mutant. TCA cycle enzymes such as citrate synthase and malate dehydrogenase were repressed in the *poxB* mutant when the cells were cultivated in LB medium. The pyruvate oxidase mutation also resulted in the activation of glucose-6-phosphate dehydrogenase and acetyl-CoA synthetase. All these results suggest that pyruvate oxidase is not only a stationary-phase enzyme as previously known, and that the removal of the *poxB* gene affects the central metabolism at the enzyme level in *E. coli*.

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Introduction

The central metabolic pathways supply the precursors for biosynthesis and provide the source for energy and reducing power in the cell. The perturbation in the specific gene expression affects the central metabolic network and thus affects the activities of the enzymes involved (Peng et al. 2004; Siddiquee et al. 2004; Vemuri et al. 2005; Li et al. 2006). Based on this experimental phenomenon, the variations of the enzyme activities in the central metabolic pathways can help understand the regulation mechanism of the cell (Peng and Shimizu 2003; Siddiquee et al. 2004; Peng et al. 2004; Li et al. 2006).

In *Escherichia coli*, pyruvate is a key intermediate in catabolic and biosynthetic pathways. As shown in Fig. 1, under aerobic conditions, pyruvate is metabolized primarily by the pyruvate dehydrogenase complex (PDHc) to acetyl-CoA. Then acetyl-CoA flows into the TCA cycle by citrate synthase (CS), or it is converted to acetate by phophotransacetylase (Pta) and acetate kinase (Ack). There are two distinct pathways for the conversion between acetyl-CoA and acetate. One pathway is catalyzed by Pta–Ack, which functions primarily in a catabolic role (Brown et al. 1977). The second pathway is catalyzed by acetyl-CoA synthetase (Acs), which is considered to be catabolite-repressible (Brown et al. 1977).

Pyruvate can also be oxidatively decarboxylated to form acetate and CO_2 via pyruvate oxidase (PoxB, encoded by *poxB* gene) in *E. coli*, which is reported to

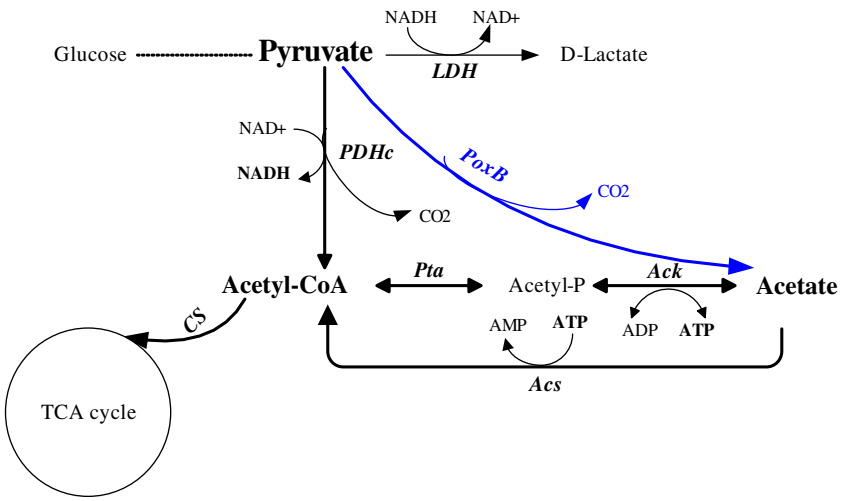

Fig. 1 The pathways of pyruvate and acetate metabolism in *E. coli* under aerobic conditions

be a non-essential and stationary-phase enzyme with uncertain physiological function (Chang and Cronan 1983; Grabau and Cronan 1984; Chang et al. 1994). Abdel-Hamid et al. (2001) demonstrated the importance of PoxB in PDHc-null *E. coli* at low dilution rates in the continuous cultivation. They also proposed that PoxB makes a significant contribution to the aerobic growth efficiency of *E. coli* and that the conversion of pyruvate to acetyl-CoA via acetate by PoxB results in the waste of energy due to the extra utilization of 1 mol ATP without producing NADH as shown in Fig. 1. Recently, Vemuri et al. (2005) studied the physiological response of *E. coli* central metabolism to the expression of heterologous pyruvate carboxylase in the presence and absence of PoxB on the gene expression level, and revealed the role of PoxB in the pyruvate node which is important in respiration and has some impact on the acetate overflow during aerobic growth. In the present study, we investigated the effect of *poxB* gene knockout on the fermentative characteristics of *E. coli* in the synthetic and LB medium, and also studied the metabolic response of *E. coli* central metabolism to *poxB* gene knockout on the enzyme level.

Materials and methods

Strains and culture conditions

E. coli BW25113 ($lacI^q$ $rrnB_{T14}$ $\Delta lacZ_{WJ16}$ *hsdR514* $\Delta araBAD_{AH33}$ $\Delta rhaBAD_{LD78}$) was used as the parent strain for the present study, and its *poxB* gene knockout mutant *E. coli* JW0855 was used as developed by Mori et al. (www.ttck.keio.ac.jp/IAB/english/research/index.htm) by one-step inactivation of chromosomal *poxB* gene using PCR primers (Datsenko and Wanner 2000; Kitagawa et al. 2005; Baba et al. 2006). The strains were stored frozen at –80 °C in Luria broth (LB: 10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, 10 g of NaCl per liter) containing 50% (v/v) glycerol. All inocula were cultivated in LB medium overnight, centrifuged, and resuspended in the corresponding experimental media. The culture media used were the synthetic medium (containing 10 g glucose/L) and LB medium (containing 11 g glucose/L). The synthetic medium contained 48 mM Na_2HPO_4, 22 mM KH_2PO_4, 10 mM NaCl, and 30 mM $(NH_4)_2SO_4$. The following components were filter sterilized and then added (per liter of final medium): 1 ml of 1 M $MgSO_4$, 1 ml of 0.1 mM $CaCl_2$, 1 ml of 1 mg of vitamin B_1 per liter, and 10 ml of trace element solution containing (per liter) 0.55 g of $CaCl_2$, 1 g of $FeCl_3$, 0.1 g of $MnCl_2{\cdot}4H_2O$, 0.17 g of $ZnCl_2$, 0.043 g of $CuCl_2{\cdot}2H_2O$, 0.06 g of $CoCl_2{\cdot}6H_2O$ and 0.06 g $Na_2MoO_4{\cdot}2H_2O$. Batch cultivations were carried out using a 2-L fermentor where the temperature was kept constant at 37 °C, and the pH was maintained at 7.0 ± 0.1 by automatic titration with 2 M NaOH or 2 M HCl. The oxygen supply to the culture was attained by controlling the stirring speed (350 rev/min) at a constant air-flow of 1 L/min.

Measurements of biomass and extracellular metabolite concentrations

Cell concentrations were estimated as the optical density of the culture at 600 nm using a spectrophotometer (Ubet-30, Jasco Co., Tokyo, Japan), and were then converted to dry cell weight (DCW) per liter according to the calibration curve between OD_{600} and DCW obtained for each strain under each cultivation condition. Glucose concentration was estimated using a Wako glucose test kit (Wako Co., Osaka, Japan).

Acetate concentration was estimated using an acetic acid kit (Boehringer Co., Mannheim, Germany). Pyruvate concentration was estimated using a pyruvate kit (F. Hoffmann-La Roche Ltd., Basel, Switzerland). O_2 and CO_2 concentrations in the bioreactor off-gas were estimated by the off-gas analyser (LX-750, Iijima Electronics Co., Japan).

Enzyme assays

Samples of the cultures were taken during the exponential growth phase to measure the levels of the following enzymes: glucokinase (Glk), glucose-6-phosphate isomerase (Pgi), 6-phosphofructosekinase (Pfk), fructose-1, 6-bisphosphatase (Fdp), fructose bisphosphate aldolase (Fba), CS, $NADP^+$-specific isocitrate dehydrogenase (ICDH), malate dehydrogenase (MDH), glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), PEP carboxylase (Ppc), $NADP^+(NAD^+)$-specific malic enzyme (Mez), lactate dehydrogenase (LDH), Ack, Acs. The preparations and the assay conditions of these enzymes are described elsewhere (Peng and Shimizu 2003; Li et al. 2006). Each measurement was performed at least three times.

Results and discussion

Effect of *poxB* gene knockout on the cell growth characteristics

Aerobic batch cultivations were carried out in the synthetic and LB media to investigate the effect of *poxB* gene knockout on the cell growth (Figs. 2 and 3). In the synthetic medium, the specific glucose uptake rate was a little lower in the *poxB* mutant than that in the parent strain as shown in Fig. 2a, where the maximum specific glucose uptake rate was 1.62 h^{-1} for the former and 1.85 h^{-1} for the latter. The *poxB* mutant showed slower growth as compared to its parent strain, where the maximum specific growth rate of the *poxB* mutant was 0.64 h^{-1}, while that of the parent strain was 0.71 h^{-1}. The biomass yield was also slightly reduced in the absence of PoxB in *E. coli* as shown in Fig. 2a. The acetate production rate was lower for the *poxB* mutant. Carbon dioxide evolution rates (CER) of the parent strain and its *poxB* mutant are shown in Fig. 2b. The CO_2 yield of the *poxB* mutant was 0.44 g/g (CO_2/glucose), and it was greater than that of the parent strain (0.38 g/g). The profile of the oxygen uptake rates (OUR) was similar with that of CER data.

The decreased glucose uptake rate in the *poxB* mutant indicated the decreased glycolytic flux in it,

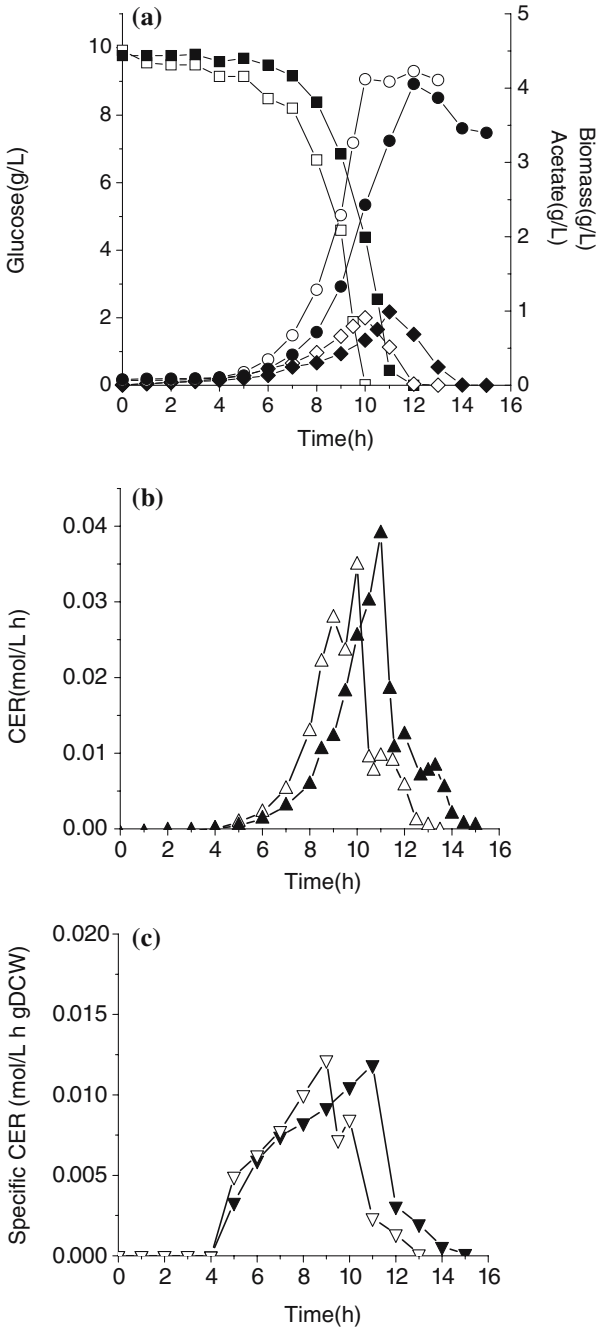

Fig. 2 Aerobic batch cultivation of *E. coli* BW25113 (empty symbols) and *E. coli* JW0855 (solid symbols) using glucose as carbon source in synthetic medium. (**a**) Glucose concentration (□, ■); Biomass concentration (○,●); Acetate concentration (◇,◆); (**b**) Carbon dioxide evolution rate (CER) (Δ,▲); (**c**) Specific CER(∇,▼)

which might be caused by the down-regulation of the glycolytic enzyme activities as will be shown later in Fig. 4. It was reported that acetate production in *E. coli* is generally attributed to the ''overflow metabolism'' (El-Mansi and Holms 1989; Han et al. 1992), and acetate formation could be reduced by decreasing

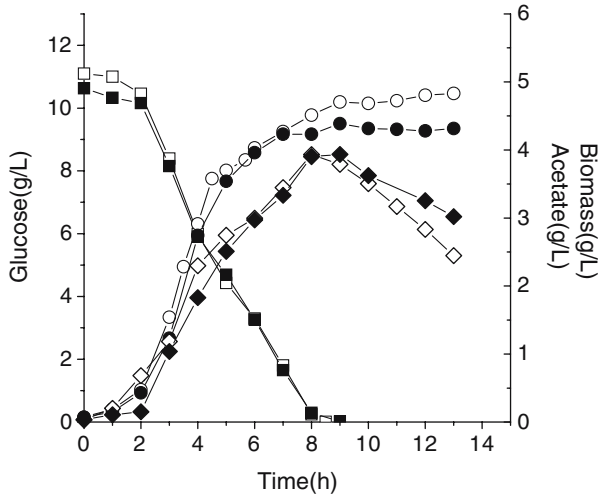

Fig. 3 Aerobic batch cultivation of *E. coli* BW25113 (empty symbols) and *E. coli* JW0855 (solid symbols) using glucose as carbon source in LB medium. Glucose concentration (□, ■); Biomass concentration (○,●); Acetate concentration (◇,◆)

the specific glucose uptake rate. Thus, the decreased glycolytic flux might be one reason for the lower acetate formation rate in the *poxB* mutant. In addition, since PoxB is an acetate-generating enzyme, its deficiency might be another reason for the lower acetate formation rate in the mutant.

Figure 3 shows the growth characteristics and acetate formation of these two strains cultivated in LB medium containing glucose as a carbon source. The final biomass was lower for the *poxB* mutant as compared to the parent strain. We also measured the extracellular pyruvate concentration, but its concentration was very low in the culture broths of both strains. The acetate production rate of the *poxB* mutant was slightly lower, however, it is shown that the amount of acetate production was not affected much in the *poxB* mutant, which might be due to the co-effect of deficiency of PoxB, decreased glycolytic flux and down-regulation of the TCA cycle in the

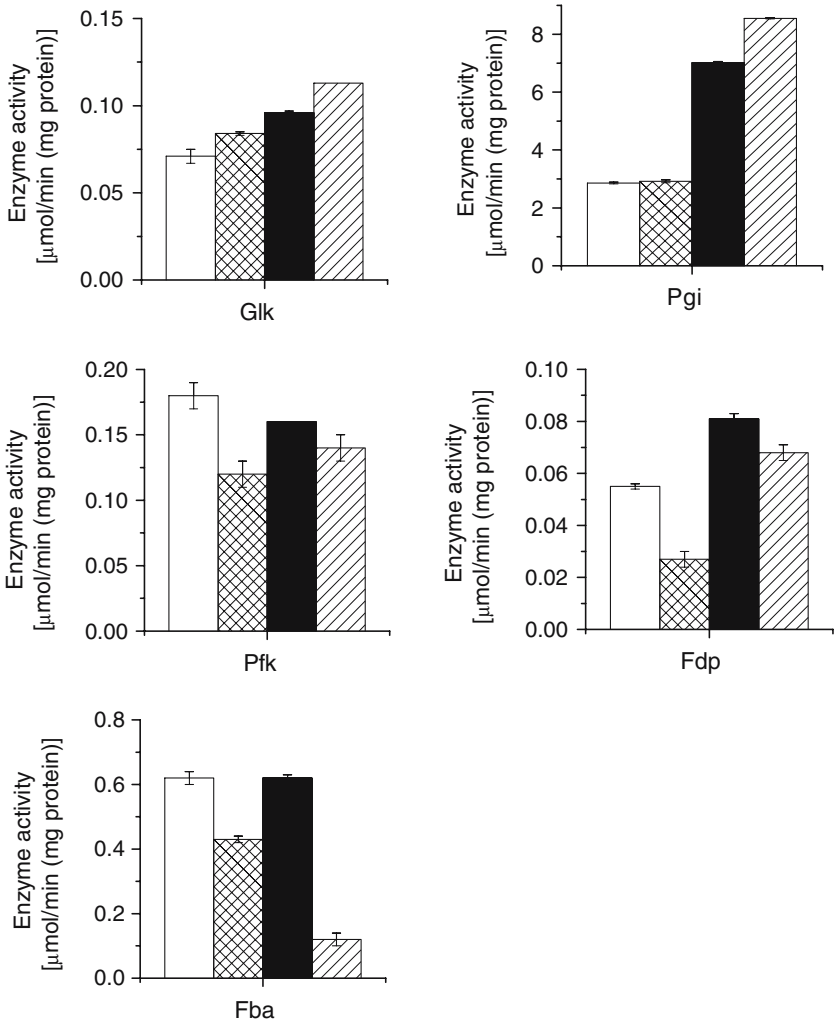

Fig. 4 Glycolysis/gluconeogenesis enzyme activities of *E. coli* BW25113 and *E. coli* JW0855 cultivated in synthetic and LB medium. *E. coli* BW25113 in synthetic medium (□); *E. coli* JW0855 in synthetic medium (▨); *E. coli* BW25113 in LB medium (■); *E. coli* JW0855 in LB medium (▨)

mutant, as will be shown later in the enzyme assay section.

Effect of *poxB* gene knockout on the enzyme activities

Since enzymes play important roles in controlling the distribution of metabolic flux in the cell, we measured the activities of the enzymes involved in the central metabolic pathways, such as the glycolysis/gluconeogenesis and pentose phosphate pathways, which are two trunk routes of intermediary sugar metabolism in *E. coli*; the TCA cycle, which is responsible for the total oxidation of acetyl-CoA and whose intermediates are required in the biosynthesis of several amino acids; the anaplerotic reactions, which replenish the intermediates of the TCA cycle.

The statistical significance of the difference between the means of enzyme activities of the two strains was assessed using the independent *t*-test. $p < 0.05$ was considered statistically significant.

Glycolysis/gluconeogenesis enzymes

As shown in Fig. 4, the removal of PoxB increased the Glk activity ($p < 0.01$ for both media) but reduced the activities of some other glycolytic enzymes such as Pfk ($p < 0.01$ for both media), Fdp ($p < 0.01$ for both media), and Fba ($p < 0.01$ for both media). Pgi activity did not show much difference between the two strains in the synthetic medium ($p > 0.05$). Although Glk activity was up-regulated in the *poxB* mutant, the removal of PoxB decreased the expression of PTS genes in *E. coli* (Vemuri et al. 2005). Pyruvate oxidase is an enzyme for pyruvate assimilation, and its absence results in the accumulation of intracellular pyruvate (Chang and Cronan 1984; Carter and Gennis 1985). Since glucose uptake via PTS was accompanied by the production of pyruvate, utilizing Glk as another glucose uptake enzyme results in less pyruvate production. The decreased PTSs and increased Glk activities caused slightly decreased glucose uptake rate in the *poxB* mutant. Pfk is a rate-limiting enzyme in glycolysis, and also *fbaA* gene expression was found to be positively related with the glycolytic flux (Tao et al. 2001). Their down-regulation was consistent with the lower glycolytic flux in the *poxB* mutant. In addition, Pgi appeared to be media-related, and its activity was over 2-fold higher in LB medium than that in the synthetic medium, while other glycolytic enzymes did not show this trend.

TCA cycle enzymes

The enzyme activity results as shown in Fig. 5 indicate that MDH is down-regulated in the *poxB* mutant as compared to the parent strain in both media ($p < 0.01$ for both media). In the synthetic medium, CS activity was almost the same in these two strains ($p > 0.05$), and ICDH was shown a slight repression in the *poxB* mutant ($p < 0.05$). In LB medium, the repression was significant in some TCA cycle enzymes such CS

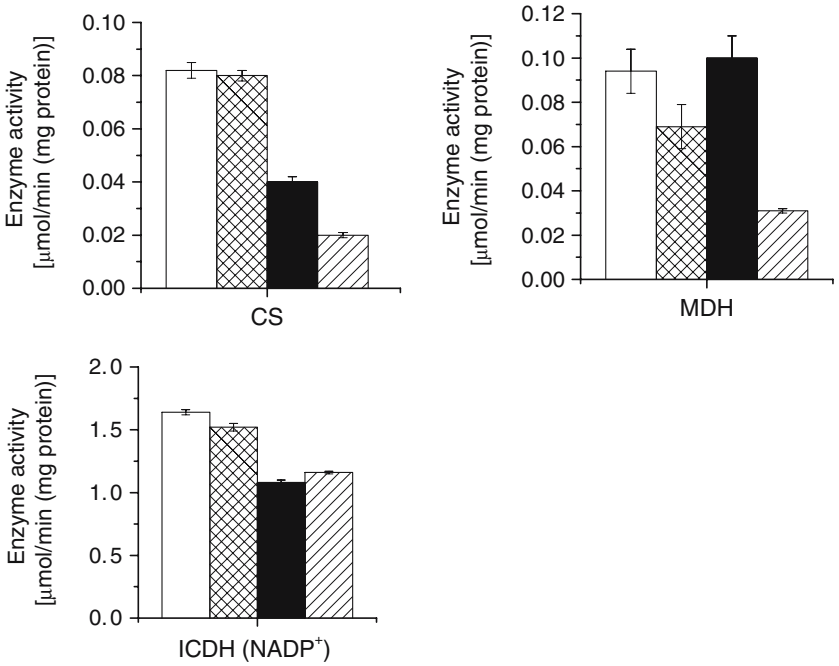

Fig. 5 TCA cycle enzyme activities of *E. coli* BW25113 and *E. coli* JW0855 cultivated in synthetic and LB medium. *E. coli* BW25113 in synthetic medium (□); *E. coli* JW0855 in synthetic medium (▨); *E. coli* BW25113 in LB medium (■); *E. coli* JW0855 in LB medium (▨)

($p < 0.01$ for both media), MDH ($p < 0.01$ for both media). The TCA cycle is a main source of the precursors for cell biosynthesis. Since LB medium directly provides peptides and amino acids, the activity of CS, a rate-limiting enzyme in the TCA cycle, was down-regulated in LB medium.

Pentose phosphate and anaplerotic pathway enzymes

The enzyme activity of the first oxidative pentose phosphate pathway enzyme such as G6PDH increased in the *poxB* mutant as compared to the parent strain, while 6PGDH did not show this trend (Fig. 6). The repression of glycolysis drove more carbon flow via G6PDH, which was confirmed by the up-regulation of this enzyme activity ($p < 0.01$ for synthetic medium, $p < 0.05$ for LB medium). Vemuri et al. (2005) found *edd* gene expression increased 2–4 times in the absence of PoxB, and the Entner–Doudoroff pathway starts the reaction from 6-phosphogluconate, which could explain why 6PGDH was not activated in the synthetic medium and down-regulated in LB medium ($p < 0.01$) in the *poxB* mutant while G6PDH was activated in the mutant.

Figure 7 indicates that Ppc was up-regulated in the absence of PoxB in the synthetic medium ($p < 0.01$). The down-regulation of PTS caused the accumulation of PEP in the cell, which may have increased the Ppc activity (Vemuri et al. 2005). While in LB medium, Ppc activity decreased in the *poxB* mutant ($p < 0.01$). Ppc operates as anaplerotic route for replenishing the TCA cycle, the down-regulation of TCA cycle might be one reason for the decrease of Ppc in LB medium.

Fermentative and acetate utilization enzymes

The up-regulation of LDH in the mutant may be due to the accumulation of endogenetic pyruvate (Causey

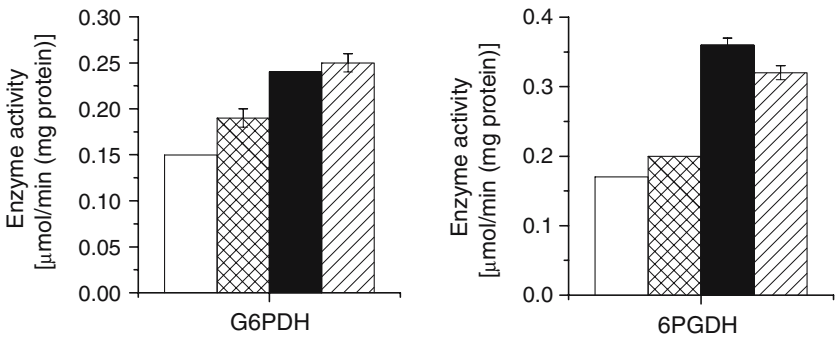

Fig. 6 Pentose phosphate pathway enzyme activities of *E. coli* BW25113 and *E. coli* JW0855 cultivated in synthetic and LB medium. *E. coli* BW25113 in synthetic medium (□); *E. coli* JW0855 in synthetic medium (⊠); *E. coli* BW25113 in LB medium (■); *E. coli* JW0855 in LB medium (▨)

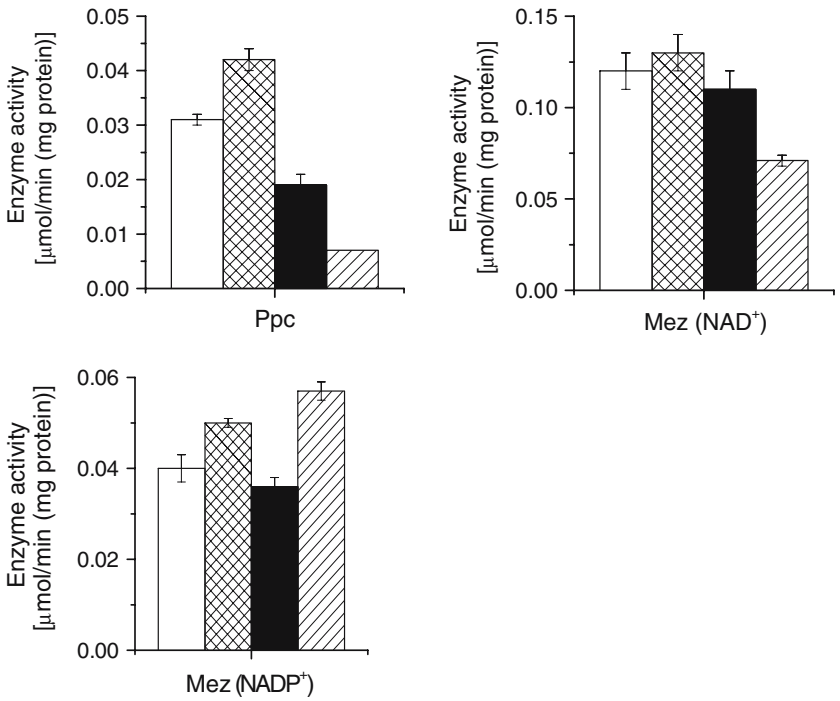

Fig. 7 Anaplerotic pathways enzyme activities of *E. coli* BW25113 and *E. coli* JW0855 cultivated in synthetic and LB medium. *E. coli* BW25113 in synthetic medium (□); *E. coli* JW0855 in synthetic medium (⊠); *E. coli* BW25113 in LB medium (■); *E.coli* JW0855 in LB medium (▨)

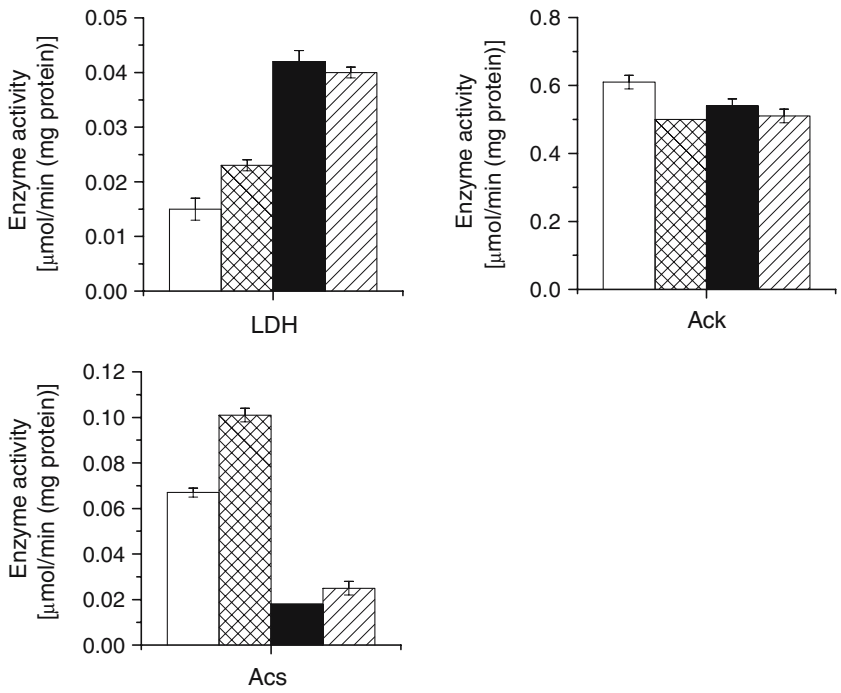

Fig. 8 Fermentative and acetate utilization enzyme activities of *E. coli* BW25113 and *E. coli* JW0855 cultivated in synthetic and LB medium. *E. coli* BW25113 in synthetic medium (□); *E. coli* JW0855 in synthetic medium (▨); *E. coli* BW25113 in LB medium (■); *E. coli* JW0855 in LB medium (▨)

et al. 2004), which is an activator of LDH (Tarmy and Kaplan 1968; Jiang et al. 2001; Li et al. 2006) (Fig. 8). In LB medium, LDH activity was higher as compared to that in the synthetic medium, while Acs activity was lower in LB medium.

In summary, *poxB* gene knockout perturbed the pyruvate node in the central metabolism, which resulted in the metabolic regulation on the enzyme level in *E. coli*, the decrease in the glucose uptake rate and biomass yield, and the increase in the carbon dioxide evolution rate and oxygen consumption rate.

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