J Ind Microbiol Biotechnol (2014) 41:1007–1015
DOI 10.1007/s10295-014-1436-1

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Modification of glycolysis and its effect on the production of L-threonine in *Escherichia coli*

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Received: 15 November 2013 / Accepted: 10 March 2014 / Published online: 27 March 2014

Abstract High concentrations of acetate, the main by-product of *Escherichia coli* (*E. coli*) high cell density culture, inhibit bacterial growth and L-threonine production. Since metabolic overflux causes acetate accumulation, we attempted to reduce acetate production by redirecting glycolysis flux to the pentose phosphate pathway by deleting the genes encoding phosphofructokinase (*pfk*) and/or pyruvate kinase (*pyk*) in an L-threonine-producing strain of *E. coli*, THRD. *pykF*, *pykA*, *pfkA*, and *pfkB* deletion mutants produced less acetate (9.44 ± 0.83, 3.86 ± 0.88, 0.30 ± 0.25, and 6.99 ± 0.85 g/l, respectively) than wild-type THRD cultures (19.75 ± 0.93 g/l). THRDΔ*pykF* and THRDΔ*pykA* produced 11.05 and 5.35 % more L-threonine, and achieved a 10.91 and 5.60 % higher yield on glucose, respectively. While THRDΔ*pfkA* grew more slowly and produced less L-threonine than THRD, THRDΔ*pfkB* produced levels of L-threonine (102.28 ± 2.80 g/l) and a yield on glucose (0.34 g/g) similar to that of THRD. The dual deletion mutant THRDΔ*pfkB*Δ*pykF* also achieved low acetate (7.42 ± 0.81 g/l) and high L-threonine yields (111.37 ± 2.71 g/l). The level of NADPH in THRDΔ*pfkA* cultures was depressed, whereas all other mutants produced more NADPH than THRD did. These results demonstrated that modification of glycolysis in *E. coli* THRD reduced acetate production and increased accumulation of L-threonine.

Keywords *Escherichia coli* · L-Threonine · Acetate · Phosphofructokinase · Pyruvate kinase

X. Xie and Y. Liang contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10295-014-1436-1) contains supplementary material, which is available to authorized users.

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Introduction

L-Threonine, an essential amino acid, is widely used in the agricultural, pharmaceutical, and cosmetics industries [25]. Currently, L-threonine is produced mainly by *Escherichia coli* (*E. coli*) fermentation [10], owing to the low manufacturing costs, variety of expression systems available, and high volumetric productivity of high cell density culture (HCDC) [11]. Acetate, the main by-product of HCDC [9], retards the growth of bacteria even at concentrations as low as 0.5 g/l. Above 5 g/l, acetate inhibits production of recombinant proteins and reduces HCDC productivity [7]. Therefore, minimizing acetate production is crucial for the maintenance of high productivity in HCDC.

Low levels of dissolved oxygen (DO) can initiate acetate generation in *E. coli* by a process known as mixed-acid fermentation. Acetate is also produced in *E. coli* when the carbon fluxes into the Embden–Meyerhof–Parnas (EMP) pathway exceeds that into the tricarboxylic acid (TCA) cycle, redirecting acetyl-CoA from the TCA cycle toward acetate [18].

Strategies aiming to reduce acetate accumulation during *E. coli* fermentation have been pursued in order to improve HCDC productivity, e.g., limiting the supply of essential nutrients can effectively control acetate production. The concentration of glucose can be tailored to the DO [14] or pH [3, 16]. However, these methods complicate the process of fermentation, limit the growth rate of *E. coli*, and reduce the production capacity of HCDC. Alternatively, acetate production can be reduced by modification of the culture medium composition [1, 33]. For example, Aristidou et al. [1] employed a medium based on fructose, instead of glucose, and successfully reduced acetate accumulation whilst simultaneously increasing protein yield by 65 %. However, these methods often increase production cost and changes in the culture medium composition can complicate downstream processing of the products of interest.

Genetic strategies to limit acetate production include deletion of *pta* [5] or *poxB* [17], genes encoding key enzymes in acetate synthesis. However, the *pta* or *poxB* mutants grow slowly on glucose and accumulate pyruvate, D-lactate, and L-glutamate instead of acetate [5, 17]. Because acetate production is linked to glucose consumption, Picon et al. [23] found that disruption of the phosphotransferase system for glucose transport eliminated acetate excretion, but reduced the maximum growth rate.

Since acetate formation is correlated with the metabolic flux overflow, we hypothesized that reducing glycolysis metabolic flux would reduce the formation of acetate. Phosphoglucose isomerase (encoded by *pgi*) catalyzes conversion of glucose-6-phosphate and fructose-6-phosphate, an essential step of the glycolysis and gluconeogenesis pathway [30, 31]. When the *pgi* gene was knocked out, the primary routes for glucose catabolism were the pentose phosphate pathway (PPP) and Entner–Doudoroff (ED) pathways, instead of glycolysis; PPP and ED were responsible for roughly 70 and 30 % of glucose catabolism, respectively [31]. However, the Δ*pgi* mutant exhibited decreased glucose uptake and growth [30]. Pyruvate kinase (PYK) catalyzes the formation of pyruvate + Mg-ATP by transferring a phosphate group from phosphoenolpyruvate (PEP) to Mg-ATP. As PEP and pyruvate connect the metabolic pathways of carbohydrates, amino acids, and lipids, PYK regulation is crucially important for glycolysis and cellular metabolism. *E. coli* expresses two PYK isoenzymes, PYKI and PYKII (encoded by *pykF* and *pykA*, respectively) [27]. In *pykF* knockout mutants, the flux of

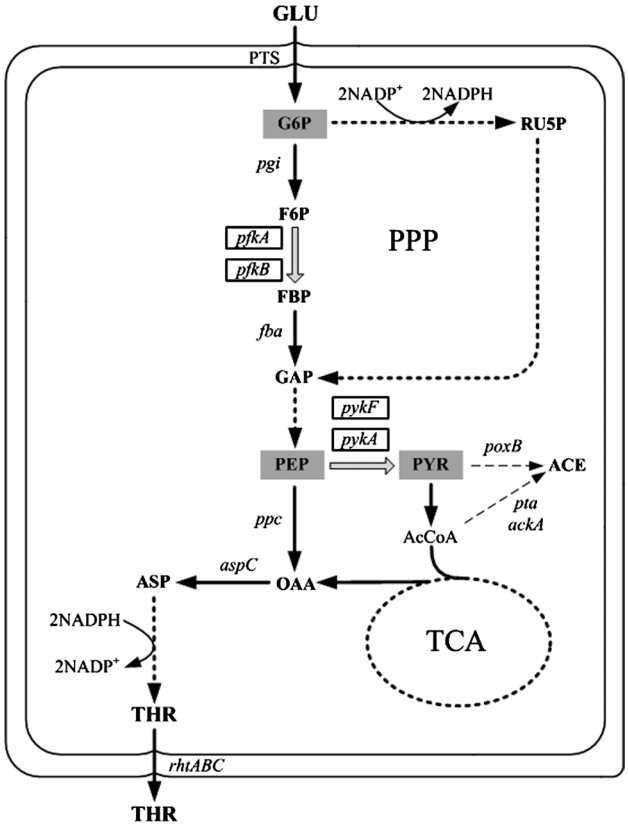

Fig. 1 The pathway of L-threonine metabolism in *E. coli* and the strategies of glycolysis modification. The *hollow arrows* indicate the weakened reaction. *Dotted lines* indicate the multiple step reactions. *Dashed lines* indicate the overflow metabolic flux that was expected to reduce. The *shaded boxes* represent the important metabolic nodes. The *colorless boxes* represent the genes that were knocked out. Genes are given in *italics*. *PPP* pentose phosphate pathway, *TCA* tricarboxylic acids cycle, *GLU* glucose, *RU5P* ribulose-5-phosphate, *G6P* glucose-6-phosphate, *F6P* fructose-6-phosphate, *FBP* fructose-1,6-bisphosphate, *GAP* glyceraldehyde-3-phosphate, *PEP* phosphoenolpyruvate, *PYR* pyruvate, *AcCoA* acetyl-CoA, *ACE* acetate, *OAA* oxaloacetate, *ASP* L-aspartate, *THR* L-threonine

EMP was reduced from 65 % in a wild-type strain to 20 % and PPP was increased from 34 to 79 % [27]. Phosphofructokinase (PFK) catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-biphosphate. PFK activity can be used as an indicator of glycolysis flux in a microorganism [30]. *E. coli* expresses two isoenzymes of PFK, PFKI and PFKII, encoded by *pfkA* and *pfkB*, respectively [30]. *pfk* deletion mutants exhibit a decreased rate of glucose consumption, with partial redirection of the metabolic flux to PPP [29].

Figure 1 illustrates the pathway by which L-threonine is metabolized in *E. coli*, in which glucose-6-phosphate, PEP, and pyruvate control the metabolic flux. In order to determine whether *pfk* and *pyk* deletion can act synergistically to reduce the glycolysis overflow flux, we deleted *pfk* and/or *pyk* in L-threonine-producing *E. coli* THRD and assessed

whether these deletions act synergistically to improve L-threonine production in fed-batch bioreactor culture.

Materials and methods

Bacterial strains, plasmids, and primers

The strains and plasmids used in this study are listed in Table 1. The L-threonine-producing strain of *E. coli*, THRD, was derived by repeated mutagenesis of *E. coli* MG1655 and stored at the Culture Collection at Tianjin University of Science and Technology (culture collection number TCCC 11825). All primers used in this study are listed in Online Resource Table 1.

Media and culture conditions

LB, SOB, and SOC media were prepared as previously described [2]. The 2-YT medium contained 16 g tryptone, 10 g yeast extract, and 5 g NaCl per liter. *E. coli* was grown at 37 °C. Seed medium contained 40 g sucrose, 15 g yeast extract, 10 ml corn steep liquor, 10 g $KH_2PO_4{\cdot}3H_2O$, 10 g $(NH_4)_2SO_4$, and 0.6 g $MgSO_4{\cdot}7H_2O$ per liter. Production medium contained 40 g glucose, 15 g yeast extract, 15 ml corn steep liquor, 10 g $KH_2PO_4{\cdot}3H_2O$, 10 g $(NH_4)_2SO_4$, 0.6 g $MgSO_4{\cdot}7H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.01 g $MnSO_4{\cdot}H_2O$, and 0.5 g sodium citrate per liter.

Table 1 *E. coli* strains and plasmids

Name	Characteristics	Source
Strains		
E. coli K12	Wild type, MG1655	This laboratory
E. coli THRD[a]	L-Threonine producer (ILE^L, AHV^r)	This laboratory
THRDΔ*pykF*	ILE^L, AHV^r, *pykF*::FRT	This study
THRDΔ*pykA*	ILE^L, AHV^r, *pykA*::FRT	This study
THRDΔ*pfkA*	ILE^L, AHV^r, *pfkA*::FRT	This study
THRDΔ*pfkB*	ILE^L, AHV^r, *pfkB*::FRT	This study
THRDΔ*pfkB*Δ*pykF*	ILE^L, AHV^r, *pfkB*::FRT, *pykF*::FRT	This study
THRDΔ*pfkB*Δ*pykA*	ILE^L, AHV^r, *pfkB*::FRT, *pykA*::FRT	This study
Plasmids		
pKD46	Am^r, λ Red-expressing vector	[8]
pKD3	Cm^r, Template vector	[8]
pCP20	Am^r, Cm^r, FLP-expressing vector	[8]

[a] The L-threonine producer *E. coli* THRD was derived by repeated mutagenesis from *E. coli* MG1655, stored at the Culture Collection at Tianjin University of Science and Technology (TCCC 11825)

Construction of *E. coli* deletion mutants

pykF was disrupted by Red helper plasmid, pKD46, as previously described [8]. Upstream and downstream regions of the DNA fragments (about 400 bp in length) were obtained by polymerase chain reaction (PCR) using the primer pairs pykF-1 and pykF-2, and pykF-3 and pykF-4 (Online Resource Table 1), respectively. The Cm^r gene (about 1,000 bp in length) was obtained using primers pKD3-up and pKD3-down with helper plasmid pKD3 as template. Splicing by overlap extension was used to fuse the fragments up- and downstream of the *pykF* and Cm^r genes with the primers pykF-1 and pykF-4. In order to remove the Cm^r gene from the integrated locus, cells were transformed with plasmid pCP20 carrying the FLP recombinant gene. Mutant sequences were verified by PCR using primers pykF-jd-up and pykF-jd-down. The same methods were used to delete the other genes.

Fermentation

Shake flask fermentation was carried out by adding 30 ml of seed culture to a 500-ml shake flask, incubated at 37 °C, and rotated at 200 rpm. Then 3 ml of seed medium at an OD_{600} of 12 was inoculated into 27 ml fermentation medium in a 500-ml shake flask. During fermentation, the pH was maintained at about 7.0 by addition of NH_4OH.

Bioreactor fermentations were performed in 5-l bioreactors (Shanghai Baoxing Bio-engineering Equipment Co., Shanghai, China) with a working volume of 3 l. The *E. coli* seed culture was grown in the same 5-l bioreactors with a working volume of 2 l to an OD_{600} of 12–14, then 1.6 l of the seed culture was discarded and the remaining fermentation culture was transferred to 5-l bioreactors for L-threonine production. Temperature was maintained at 37 °C and pH was maintained automatically between 6.7 and 7.0 with NH_4OH. The glucose concentration was stabilized at 5 g/l with addition of 800 g/l where necessary. Dissolved oxygen tension was maintained at 30–40 %.

Analytics

Biomass was monitored by a spectrophotometer at a wavelength of 600 nm and one unit of OD_{600} was considered to be equal to 0.37 g of dry cell weight (DWC). Glucose concentration was measured with a biosensor analyzer (SBA-40B, Biology Institute of Shandong Academy of Sciences, China).

For determination of L-threonine concentration, the supernatants were diluted twofold with doubly distilled water, and derivatized with 1-fluoro-2,4-dinitrobenzene, and measured by HPLC (1200 series, Agilent Technologies, USA) equipped with an Agilent ZORBAX Eclipse

AAA column (4.6 mm × 150 mm, 5 μm). Elution was performed using a gradient of reagent A (50 % acetonitrile v/v) and reagent B (0.05 M CH_3COONa, pH 6.4), and fed at a constant flow rate of 1.0 ml/min. UV absorption was measured at 360 nm and the column temperature was maintained at 33 °C .

Organic acids were also quantified by HPLC equipped with a Bio-Rad Aminex HPX-87H column (300 mm × 7.8 mm, L × ID), and 0.005 M H_2SO_4 as a mobile phase with a flow rate of 0.5 ml/min. UV absorption was determined at 210 nm and the column temperature was maintained at 30 °C.

NADPH assays

Unless otherwise indicated, all procedures were carried out at 4 °C. Samples of 50 ml were taken at 6, 12, 18, and 24 h from the 5-l fed-batch fermentation culture, centrifuged, washed twice with 50 mM (pH 7.2) KH_2PO_4, resuspended in 50 mM (pH 7.2) KH_2PO_4 allowing 5 ml buffer per 0.1 g (wet weight) of cells. The cell suspension was divided into 30-ml aliquots to which 100 μl of lysozyme was added. After incubation at 37 °C for 30 min, the mixtures were sonicated for 5 s in 4 s intervals for a total of 15 min, then centrifuged at 7,000 rpm for 10 min. All supernatants were stored at −80 °C. The relative quantity of NADPH in each supernatant was quantified by a previously described enzymatic method [34].

Results

Effect of *pyk* and *pfk* deletion on L-threonine production

To investigate whether *pyk* and *pfk* deletions influence biomass and L-threonine production in *E. coli* THRD, we carried out shake flask fed-batch fermentations. After fermentation for 28 h, *E. coli* THRDΔ*pfkA* produced significantly less biomass (6.92 ± 0.45 g DCW/l) and L-threonine (7.36 ± 0.71 g/l) than *E. coli* THRD did (10.36 ± 0.42 g DCW/l, and 30.45 ± 1.03 g/l, respectively, both $P < 0.05$; Table 2). However, neither biomass nor L-threonine produced was significantly altered in THRDΔ*pykF*, THRDΔ*pykA*, THRDΔ*pfkB*, THRDΔ*pfkB*Δ*pykF*, or THRDΔ*pfkB*Δ*pykA* (Table 2), indicating that under shake flask fermentation conditions, the deletion of *pfkA* significantly impaired the growth of THRD and reduced the L-threonine yield, whereas deletion of *pyk* or/and *pfkB* did not significantly influence L-threonine accumulation with normal bacterial growth.

Fermentation of *E. coli* THRD with *pyk* deletion

We scaled up culture of the mutants in 5-l fed-batch fermentations, as indicated in Fig. 2 and Table 3. The results showed that *pyk* deletions did not affect the bacterial growth or the rate of glucose consumption (Fig. 2). Using an in silico method, Rodríguez-Prados et al. [26] predicted that the disruption of PYK could improve L-threonine production. We observed that L-threonine accumulation was equivalent in THRD and THRDΔ*pyk* cultures until 14 h of fermentation; thereafter, the L-threonine concentration in THRDΔ*pykF* and THRDΔ*pykA* cultures exceeded that in THRD cultures (Fig. 2). After 28 h THRDΔ*pykF* and THRDΔ*pykA* cultures contained 11.05 and 5.35 % more L-threonine than THRD cultures did (112.57 ± 2.82 and 106.79 ± 2.80 vs. 101.37 ± 2.79 g/l, respectively; Fig. 2; Table 3; $P < 0.05$). The L-threonine yields on glucose by THRDΔ*pykF* and THRDΔ*pykA* were 10.91 and 5.60 % higher than that of THRD (0.38, 0.36 vs. 0.34 g/g, Table 3; $P < 0.05$).

Pyruvate availability decreased as a result of reduced glycolysis metabolic flux and increased anaplerotic flux in the *pyk* mutant, and low flux ratios resulted in low lactate and acetate production [28]. We also observed low lactate and acetate production in fermentations where the glucose concentration was maintained at 5 g/l. During the fermentation process, acetate levels were consistently lower in THRDΔ*pyk* cultures than THRD cultures (Fig. 2; Table 3). After 28 h, THRDΔ*pykF* and THRDΔ*pykA* cultures accumulated 9.44 ± 0.83 and 3.86 ± 0.88 g/l acetate, respectively, 52.20 and 80.45 % less than THRD cultures did (19.75 ± 0.93 g/l; Fig. 2; Table 3; $P < 0.05$). In addition, the Y_{Ace}/Y_{Thr} of the THRD, THRDΔ*pykF*, and THRDΔ*pykA* cultures were 19.48, 8.38, and 3.61 %, respectively, suggesting that the carbon source had been directed to L-threonine more effectively in the mutants than in the control strain, resulting in better utilization of carbon in the mutants for L-threonine production.

Fermentation of *E. coli* THRD with *pfk* deletion

E. coli possesses two PFK isozymes, PFKI and PFKII (encoded by *pfkA* and *pfkB*, respectively), but PFKI

Table 2 Effect of *pyk* or/and *pfk* deletion on L-threonine production

Strain	Final culture biomass (g DCW/l)	Final L-threonine concentration (g/l)
THRD	10.36 ± 0.42	30.45 ± 1.03
Δ*pykF*	10.42 ± 0.47	34.31 ± 0.85
Δ*pykA*	10.88 ± 0.39	33.40 ± 0.93
Δ*pfkA*	6.92 ± 0.45*	7.36 ± 0.71*
Δ*pfkB*	10.34 ± 0.44	32.23 ± 0.89
Δ*pfkB*Δ*pykF*	10.28 ± 0.56	33.67 ± 1.02
Δ*pfkB*Δ*pykA*	10.30 ± 0.49	33.53 ± 0.69

* Indicates significant difference from the data of THRD ($P < 0.05$)

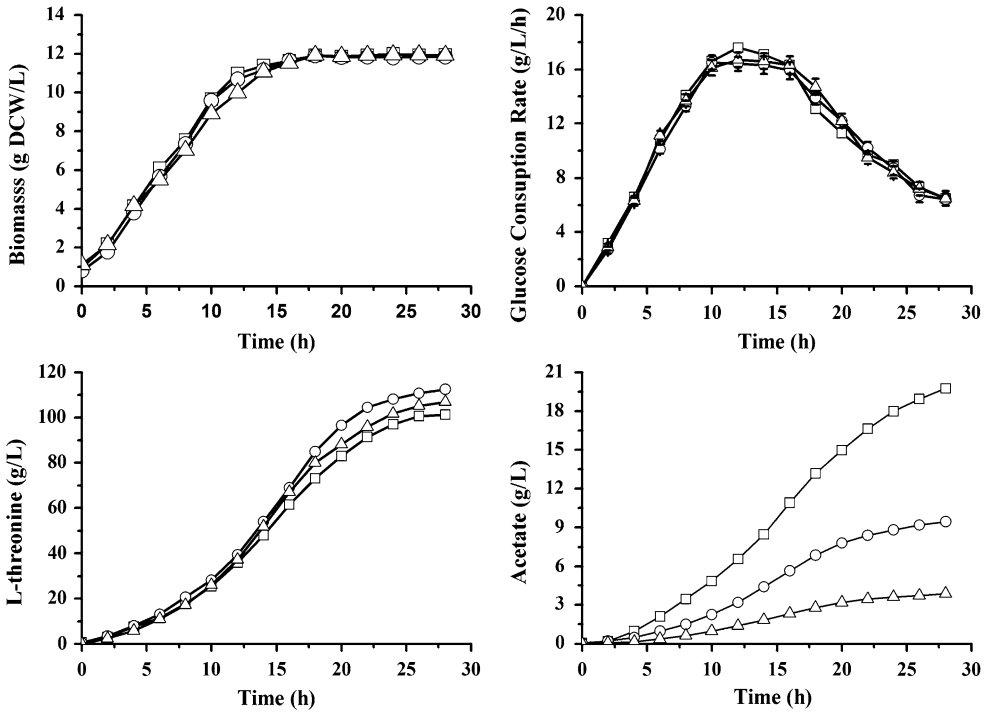

Fig. 2 Biomass, glucose consumption rate, L-threonine production, and acetate concentration of *E. coli* THRD (*squares*), *E. coli* THRDΔ*pykF* (*circles*), and *E. coli* THRDΔ*pykA* (*triangles*) in the fed-batch fermentation

Table 3 Recombinant strain L-threonine and acetate production after 28 h of fermentation

	Biomass (g DCW/l)	L-Threonine (g/l)	L-Threonine yield on glucose (g/g)	Acetate (g/l)	Acetate yield on glucose (g/g)	Y_{Ace}/Y_{Thr} (%)
THRD	11.94 ± 0.59	101.37 ± 2.79	0.339	19.75 ± 0.93	0.066	19.48
Δ*pykF*	11.83 ± 0.52	112.57 ± 2.82*	0.376*	9.44 ± 0.83*	0.031*	8.38*
Δ*pykA*	11.92 ± 0.55	106.79 ± 2.80*	0.358*	3.86 ± 0.88*	0.013*	3.61*
Δ*pfkA*	8.79 ± 0.49*	21.18 ± 2.61*	0.318*	0.30 ± 0.25*	0.004*	1.41*
Δ*pfkB*	11.78 ± 0.63	102.28 ± 2.80	0.340	6.99 ± 0.85*	0.023*	6.83*
Δ*pfkB*Δ*pykF*	11.89 ± 0.59	111.37 ± 2.71*	0.369*	7.42 ± 0.81*	0.025*	6.66*
Δ*pfkB*Δ*pykA*	11.73 ± 0.64	105.97 ± 2.47*	0.353*	7.09 ± 0.79*	0.024*	6.69*

* Indicates significant difference from the data of THRD ($P < 0.05$)

accounts for 90 % of the total enzyme activity. Since PFK is important for glucose catabolism via both glycolysis and PPP, the growth rate and glucose uptake rate of *E. coli pfkA* mutant was considerably reduced [19]. Similar results were observed in our 5-l fermentation culture, although growth of the THRDΔ*pfkB* mutant was not inhibited (Fig. 3).

pfkA and *pfkB* deletions have been shown to increase the production of NADPH-dependent products [30] including L-threonine [10]. We speculated that deletion of *pfkA* or *pfkB* may increase L-threonine production. Interestingly, we found that the THRDΔ*pfkB* L-threonine production and yield on glucose did not significantly differ from that of THRD (101.37 ± 2.79 g/l and 0.34 g/g, Fig. 3; Table 3), whereas THRDΔ*pfkA* produced 79.11 and 0.06 % less L-threonine and L-threonine yield on glucose than THRD did (Fig. 3; Table 3; $P < 0.05$). Decreased glucose consumption rate seemed to result in low productivity of THRDΔ*pfkA*.

Low acetate concentrations were also found in the THRDΔ*pfk* mutants, confirming a previous report [21]. After 28 h THRDΔ*pfkA* and THRDΔ*pfkB* accumulated significantly less acetate (0.30 ± 0.25 and 6.99 ± 0.85 g/l, respectively) than THRD did (19.75 ± 0.93 g/l, Table 3; $P < 0.05$) and achieved lower acetate yields on glucose (0.004 and 0.023 g/g, respectively) than THRD did (0.066 g/g, Table 3; $P < 0.05$). Additionally, the Y_{Ace}/Y_{Thr} values of THRDΔ*pfkA* and THRDΔ*pfkB* were 1.41 and 6.83 %, much lower than that of THRD (19.48 %; $P < 0.05$), suggesting that, among the three strains, the carbon flux in these mutants was more efficiently directed to L-threonine.

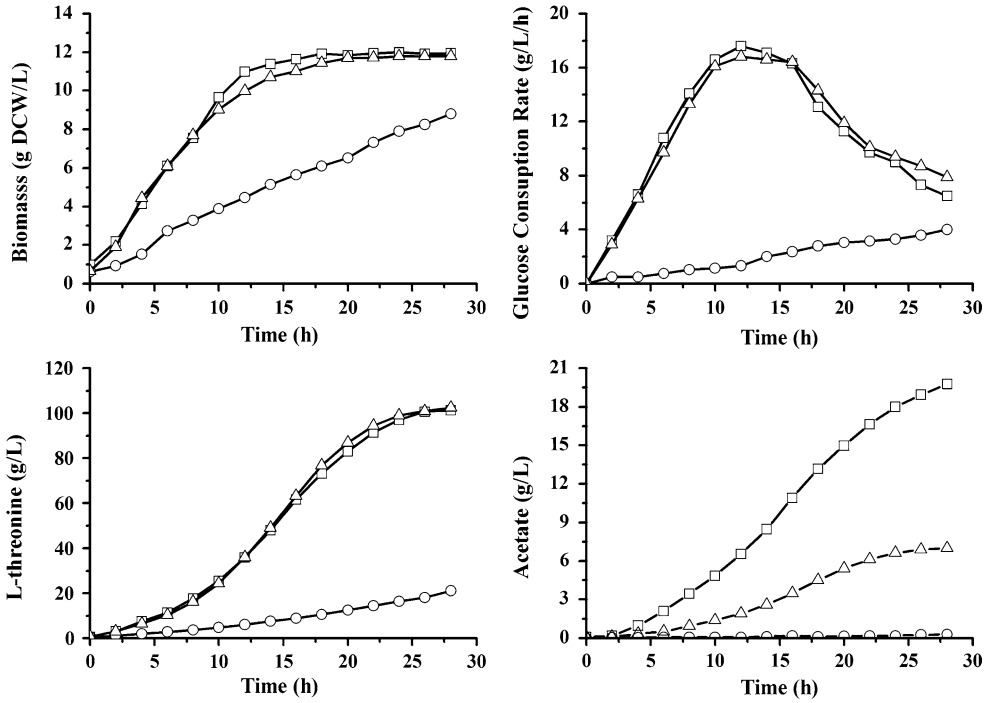

Fig. 3 Biomass, glucose consumption rate, L-threonine production, and acetate concentration of *E. coli* THRD (*squares*), *E. coli* THRDΔ*pfkA* (*circles*), and *E. coli* THRDΔ*pfkB* (*triangles*) in the fed-batch fermentation

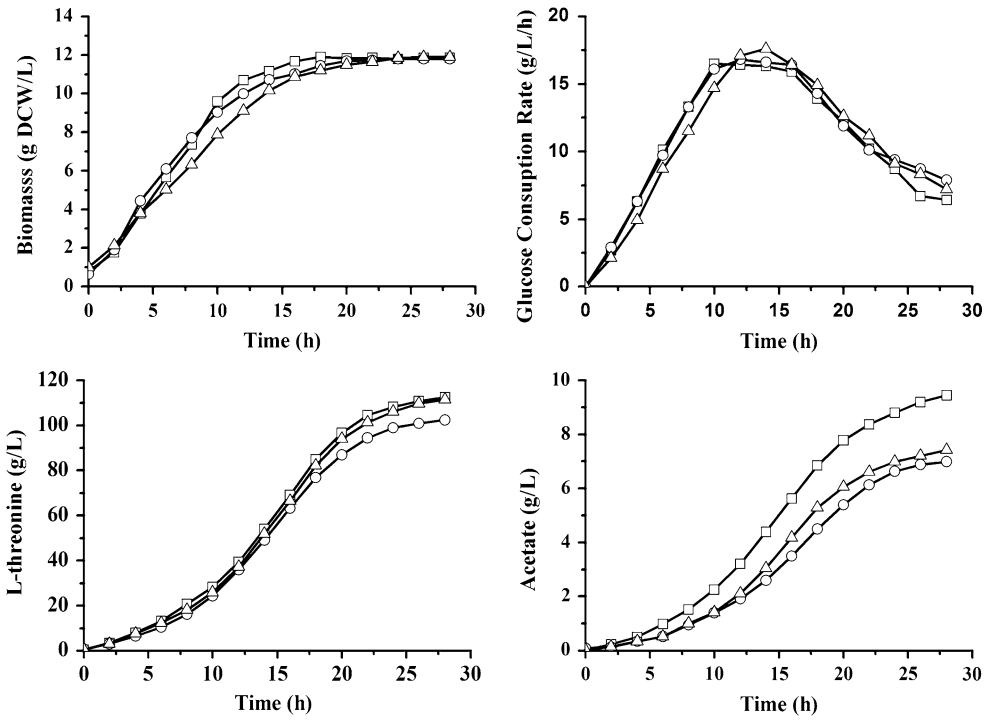

Fig. 4 Biomass, glucose consumption rate, L-threonine production, and acetate concentration of *E. coli* THRDΔ*pykF* (*squares*), *E. coli* THRDΔ*pfkB* (*circles*), and *E. coli* THRDΔ*pfkB*Δ*pykF* (*triangles*) in the fed-batch fermentation

Fermentation of *E. coli* with *pfkB* and *pyk* dual deletion

In order to determine whether the simultaneous deletion of PFK and PYK would further redirect glycolysis flux, and whether these deletions would act synergistically to affect L-threonine and acetate production, we produced the dual mutants THRDΔ*pfkB*Δ*pykF* and THRDΔ*pfkB*Δ*pykA*. During the first 15 h of fermentation, the dual mutant THRDΔ*pfkB*Δ*pykF* grew more slowly than the single mutants and consumed less glucose (Fig. 4). However, after 28 h, the biomass and glucose consumption of both dual mutants were similar to that of single mutants (Table 3).

Though the dual mutant THRDΔ*pfkB*Δ*pykF* exhibited a slightly slower growth rate and lower glucose consumption

Table 4 NADPH levels in *E. coli* control strain and deletion mutants during the fermentation

Strains	6 h	12 h	18 h	24 h
THRD	512.72 ± 7.59	491.45 ± 6.45	428.85 ± 8.36	502.63 ± 6.43*
Δ*pykF*	709.47 ± 9.01*	642.26 ± 10.81*	620.14 ± 9.87*	646.72 ± 8.25*
Δ*pykA*	639.94 ± 7.62*	572.32 ± 8.37*	522.17 ± 8.92*	568.41 ± 7.63*
Δ*pfkA*	224.58 ± 3.71*	201.63 ± 4.32*	182.98 ± 4.03*	209.71 ± 3.98*
Δ*pfkB*	562.37 ± 6.82	509.44 ± 7.35	442.27 ± 8.61	517.19 ± 8.05
Δ*pfkB*Δ*pykF*	692.33 ± 8.25*	651.92 ± 8.92*	611.29 ± 9.01*	636.84 ± 7.93*
Δ*pfkB*Δ*pykA*	668.72 ± 8.26*	559.81 ± 7.46*	530.33 ± 8.24*	607.36 ± 9.12*

NADPH concentration: nmol/(g DCW)

* Indicates significant difference from the data of THRD ($P < 0.05$)

rate than THRDΔ*pykF* and THRDΔ*pfkB* in the initial stage of fermentation, the dual mutant produced a similar concentration of L-threonine to THRDΔ*pykF* (111.37 ± 2.71 and 112.57 ± 2.82 g/l, respectively; Fig. 4; Table 3), and THRDΔ*pfkB*Δ*pykF* L-threonine production was 8.89 % higher than that of THRDΔ*pfkB* (102.28 ± 2.80 g/l; $P < 0.05$). Nevertheless, the L-threonine yield on glucose of THRDΔ*pfkB*Δ*pykF* (0.37 g/g) was 1.86 % lower than that of THRDΔ*pykF* (0.38 g/g), which was 8.53 % higher than that of THRDΔ*pfkB* (0.34 g/g; $P < 0.05$). THRDΔ*pfkB*Δ*pykA* behaved similarly to THRDΔ*pykA* (Table 3).

We expected the dual mutants to produce less acetate; however, between 14 and 28 h of fermentation, slightly more acetate was produced in the mutants than in THRDΔ*pfkB* (Fig. 4). After 28 h THRDΔ*pfkB*Δ*pykF* produced 21.40 % less acetate than THRDΔ*pykF* (7.42 ± 0.81 vs. 9.44 ± 0.83 g/l; $P < 0.05$), but 6.15 % more than THRDΔ*pfkB* did (7.42 ± 0.81 vs. 6.99 ± 0.85 g/l). The acetate yield on glucose in THRDΔ*pfkB*Δ*pykF* (0.025 g/g) was slight lower than in THRDΔ*pykF* (0.031 g/g), and similar to that in THRDΔ*pfkB* (0.023 g/g). However, the Y_{Ace}/Y_{Thr} calculated for the dual mutant was 20.52 % less than that for THRDΔ*pykF* ($P < 0.05$, Table 3), suggesting that in the dual mutant more carbon flux was directed to the L-threonine, leading to more efficient L-threonine production. This observation may be attributed to a number of factors. Firstly, the distribution effect of *pfkB* deletion on carbon flux might result in lower acetate flux in the dual mutant than in THRDΔ*pykF*; secondly, in the dual mutant, deletion of *pykF* could drive carbon sources to L-threonine production more efficiently and increase the consumption of glucose from the fermentation medium, resulting in higher carbon metabolic flux than in THRDΔ*pfkB*. In addition, THRDΔ*pfkB*Δ*pykA* mutants accumulated similar levels of acetate to THRDΔ*pfkB* (Table 3).

Effects of *pyk* and *pfk* deletions on NADPH formation

In *E. coli pfk* deletion mutants, high flux would be redirected through PPP [30]. In the *pykF* mutant flux through PPP was found to be upregulated [28]. Moreover, in *E. coli* and many other organisms, the majority of NADPH is formed by the PPP [30]. Therefore, deletion of *pfk* or *pyk* is expected to increase NADPH synthesis, increasing production of NADPH-dependent products [29], including L-threonine [10]. We measured the NADPH concentrations in control and deletion mutant cultures at four time points during fermentation.

After 12 and 18 h of fermentation, we observed a decrease in NADPH concentration (Table 4), which could be attributed to higher NADPH consumption during L-threonine biosynthesis, a process dependent on aspartyl semialdehyde dehydrogenase and homoserine dehydrogenase [10]. After 24 h of fermentation, the NADPH concentrations in the control and all mutants increased, indicating that the rate of specific L-threonine production declined in aging bacteria with reduced metabolic activity.

In addition, THRDΔ*pykF*, THRDΔ*pykA*, and THRDΔ*pfkB* produced more NADPH than THRD did (Table 4), corroborating previous reports that the *pykF*, *pykA*, and *pfkB* deletions could increase PPP flux and facilitate more efficient NADPH production [28, 30]. Double deletion of *pfkB* and *pykF* or *pykA* did not significantly alter NADPH production beyond that observed in THRDΔ*pykF* and THRDΔ*pykA*, suggesting that *pfkB* deletion did not redirect metabolic flux in the *pyk* mutant.

Discussion

Metabolic flux between glycolysis and TCA is the main source of acetate formation in *E. coli* [18], and genetic modifications targeting glycolysis can reduce acetate production [21, 24]. We examined the impact of deletion of key glycolysis enzymes on acetate and L-threonine production in *E. coli*, and were able to reduce acetate excretion and enhance L-threonine production by attenuating the metabolic flux of glycolysis.

The flux distribution at the PEP node was believed to be one of the limiting factors for the amino acid biosynthetic pathways derived from PEP (such as aspartate family and aromatic amino acids) [26]. *E. coli* and *Bacillus subtilis pyk* mutants were shown to have significantly increased metabolic flux through PEP [12] and decreased flux though acetate [12, 24, 35]. Kedar et al. [15] reported that *pyk* deletion enhanced aromatic amino acid concentration and reduced acetate accumulation. We confirmed that deletion of *pyk* in *E. coli* THRD indeed enhanced L-threonine production and reduced acetate accumulation in 5-l fermentation maintained with 5 g/l glucose. However, the increase of L-threonine yield in THRDΔ*pykF* was higher than that of THRDΔ*pykA*, probably as a result of the greater contribution of *pykF* to PYK activity; thus, *pykF* deletion enhanced more anaplerotic flux to oxaloacetate [13]. Hence, in THRDΔ*pykF*, enhanced flux to L-threonine synthesis was observed. These observations may also highlight an explanation for the difference in L-threonine yields observed between THRDΔ*pfkB*Δ*pykF* and THRDΔ*pfkB*Δ*pykA*.

pfk mutants have been shown to have higher PPP flux and lower acetate flux, enhancing accumulation of NADPH-dependent products [6, 30, 32] and reducing acetate production [21]. The synthesis of L-threonine, L-lysine, and L-valine require NADPH as cofactors [10], and increasing NADPH availability has previously been employed to improve L-lysine [4, 20] and L-valine [22] production. While we expected that deletion *pfkA* and *pfkB* in *E. coli* THRD could increase L-threonine production and decrease acetate accumulation, these changes were not observed in the two Δ*pfk* mutants, although both strains secreted less acetate. While THRDΔ*pfkB* produced similar levels of L-threonine to that of THRD, THRDΔ*pfkA* produced only roughly one-fifth of the L-threonine produced by THRD. This observation may be a result of the influence of *pfkB* deletion on the metabolic flux gap between EMP and TCA, resulting in reduced overflow flux directed to acetate. However deletion of *pfkB* did not markedly increase NADPH production, and L-threonine production was comparable to that in THRD as a result of the shortage of NADPH. These findings were consistent with the impact on L-threonine and NADPH production of *pfkB* and *pykF* dual deletion or *pykF* single deletion, which were very similar. Though the production of L-threonine was similar, Y_{Ace}/Y_{Thr} ratios were lower in THRDΔ*pfkB*Δ*pykF* than THRDΔ*pykF*. The poor growth and lower glucose consumption of THRDΔ*pfkA* might be responsible for its low L-threonine and acetate production.

Though it is well known that overflow flux is the main reason for acetate production, our results showed for the first time that simultaneous inhibition of PFK and PYK could reduce the glycolysis flux and decrease the overflow directed to acetate in *E. coli*. We found that simultaneous deletion of *pfkB* and *pykF* resulted in more efficient carbon utilization, higher L-threonine production, and lower acetate accumulation in comparison to those of single gene deletions. Impairment of carbon metabolism was observed in some deletion mutants, leading to poor bacterial growth. Hence, moderate redirection of the flux may more effectively enhance mutant growth and product formation.

Acknowledgments This research was supported by the National High Technology Research and Development Program (2012AA022102 and 2012AA02A703) and the National Basic Research Program of China (2013CB733901).

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