Appl Microbiol Biotechnol (2016) 100:2775–2784
DOI 10.1007/s00253-015-7237-2

APPLIED MICROBIAL AND CELL PHYSIOLOGY

Deletion of *poxB*, *pta*, and *ackA* improves 1,3-propanediol production by *Klebsiella pneumoniae*

Jie Lin[1] · **Yongqiang Zhang**[1] · **Danfeng Xu**[1] · **Gang Xiang**[1] · **Zongxiao Jia**[1] · **Shuilin Fu**[1] · **Heng Gong**[1]

Received: 27 August 2015 / Revised: 29 November 2015 / Accepted: 7 December 2015 / Published online: 23 December 2015

Abstract To date, few studies have focused on reducing the toxic by-product acetate during 1,3-propanediol production by *Klebsiella pneumoniae*. In this study, the effects of deleting the *poxB*, *pta*, and *ackA* genes, which are involved in the two main acetate synthesis pathways, on cell growth and 1,3-propanediol production were investigated. Although acetate synthesis via pyruvate oxidase (PoxB, encoded by *poxB*) generally seems unnecessary and wasteful, PoxB was shown to play an important role in *K. pneumoniae*. Deletion of *poxB* severely inhibited cell growth, and the *poxB* mutant exhibited an anomalously high accumulation of acetate in aerobic cultures and failed to produce an endogenous supply of carbon dioxide (CO_2) in anaerobic cultures. It is interesting that both the aerobic and anaerobic growth defects of the *poxB* mutant were corrected by further deleting *pta* and *ackA*, which blocked the other main acetate synthesis pathway. The *poxB-pta-ackA* mutant excreted less acetate and showed an excellent ability to produce 1,3-propandiol. The final 1,3-propanediol yield and concentration in a 2-L fed-batch fermentation reached 0.66 (mol/mol) and 76.8 g/L, respectively, which were 16 and 15 % greater, respectively, than those of the parent strain.

Keywords 1,3-Propanediol · Acetate · *ackA* · *Klebsiella pneumonia* · *poxB* · *pta*

✉ Heng Gong
gongheng@ecust.edu.cn

[1] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, People's Republic of China

Introduction

1,3-Propanediol (1,3-PD) is a valuable and important chemical that is widely used in polyester, cosmetic, and pharmaceutical industries (Durgapal et al. 2014; Szymanowska-Powalowska and Kubiak 2015). Because of the large amount of low-cost raw glycerol produced during biodiesel production, the biological production of 1,3-PD from glycerol has attracted significant interest recently (Khan et al. 2013; Szymanowska-Powalowska and Bialas 2014). *Klebsiella pneumoniae*, a facultative anaerobe, has the ability to produce high yields of 1,3-PD from glycerol (Celinska 2012; Yen et al. 2014).

The metabolic pathway responsible for the microbial production of 1,3-PD from glycerol has been well studied in *K. pneumoniae* (Kumar et al. 2012; Seo et al. 2009; Skraly et al. 1998; Zhuge et al. 2010). Generally, glycerol is converted through reductive and oxidative pathways (Fig. 1), the reductive branch leading to 1,3-PD production, and the oxidative branch providing the reducing power for 1,3-PD synthesis; during the fermentation process, the formation of by-products derived from oxidative branch, including acetate, lactate, and 2,3-butanediol (2,3-BD), leads to the decreases of carbon flux and reducing power towards 1,3-PD synthesis (Huang et al. 2012; Petrov and Petrova 2009; Tang et al. 2009). To improve the production of 1,3-PD in *K. pneumoniae*, various efforts have been made to delete the genes responsible for the formation of these by-products. It was found that lactate formation was blocked by deleting the *ldhA* gene (Xu et al. 2009; Zheng et al. 2008), and that 2,3-BD formation was also completely abolished by deleting the genes responsible for its synthesis (Cui et al. 2014). Because blocking 2,3-BD formation results in an extreme growth defect and a decrease in 1,3-PD production (Cui et al. 2014; Zhu et al. 2015), many high 1,3-

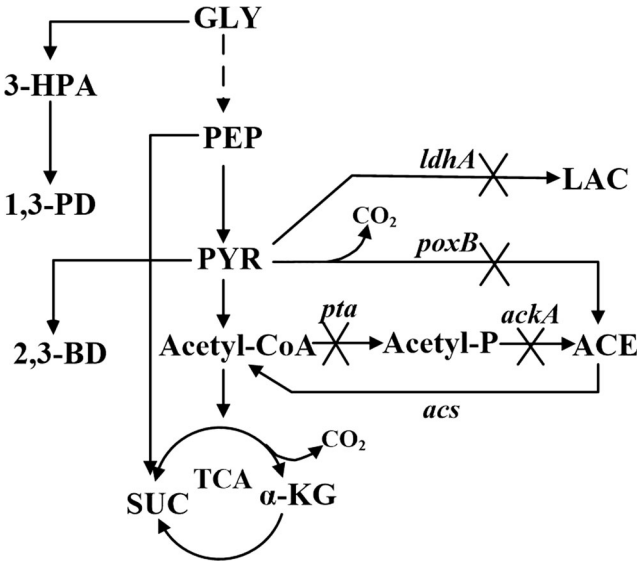

Fig. 1 Metabolic pathways of glycerol metabolism in *K. pneumoniae*. *GLY* glycerol, *PYR* pyruvate, *PEP* phosphoenolpyruvate, *3-HPA* 3-hydroxypropinaldehyde, *1,3-PD* 1,3-propanediol, *2,3-BD* 2,3-butanediol, *LAC* lactate, *ACE* acetate, *SUC* succinate, *α-KG* α-ketoglutarate, *TCA* tricarboxylic acids cycle, *Acetyl-P* acetylphosphate, *pta* encoding phosphotransacetylase, *ackA* encoding acetate kinase, *poxB* encoding pyruvate oxidase, *ldhA* encoding lactate dehydrogenase, *acs* encoding acetyl-CoA synthetase

PD producers are based on lactate-deficient (*ldhA*-deleted) *K. pneumoniae* strains, although the deletion of *ldhA* is actually more beneficial for 2,3-BD production than 1,3-PD production (Durgapal et al. 2014; Kumar et al. 2013; Xu et al. 2009).

Compared with lactate and 2,3-BD, few reports have focused on reducing the other main by-product, acetate, which is commonly considered to be more toxic than lactate and 2,3-BD (Celinska 2010). Recently, Lee et al. (2014) reported that deleting the *pta* gene decreased acetate formation in a both 2, 3-butanediol- and lactate-deficient *K. pneumoniae* strain. In *Escherichia coli*, acetate is synthesized by two major routes: the first occurs via the actions of phosphotransacetylase (Pta, encoded by the *pta* gene) and acetate kinase (AckA, encoded by the *ackA* gene), which convert acetyl-CoA to acetate and one molecule of adenosine triphosphate (ATP); the other route is via pyruvate oxidase (PoxB, encoded by the *poxB* gene), which oxidizes pyruvate to generate acetate and one molecule of CO_2 (Ashok et al. 2011). Generally, the Pta-AckA pathway is the primary one, while deletion of *poxB* seems to affect central carbon metabolism, such as the tricarboxylic acid (TCA) cycle. Deleting both pathways results in decreased cell growth and acetate formation in *E. coli* (De Mey et al. 2007; Li et al. 2012). On the other hand, acetate can be assimilated by *E. coli*, mainly through acetyl-CoA synthetase (ACS, encoded by the *acs* gene) to generate acetyl-CoA. The recycling of acetate by ACS preserves the pool of free reduced CoA (CoA-SH) and mitigates acetate overflow in aerobic *E. coli* cultures (Dittrich et al. 2005; Peebo et al. 2014). Because of its close genetic relatedness to *E. coli*, *K. pneumoniae* is expected to have the same regulatory mechanism for acetate synthesis. However, to date, no detailed study has focused on the acetate synthesis pathway, especially, its effect on 1,3-PD production and physiology of *K. pneumoniae.*

In this study, the effects of blocking the two acetate synthesis pathways, PoxB and Pta-AckA, on cell growth and 1,3-PD production in *K. pneumoniae* were investigated under different culture conditions. It was shown that deletion of *poxB* severely inhibited cell growth. Interestingly, the growth defect of the *poxB* mutant was alleviated by further deleting the Pta-AckA acetate synthesis pathway. With comprehensive analysis, the roles of PoxB and Pta-AckA in *K. pneumoniae* were represented. And, as a result, a strategy for efficient production of 1,3-PD by directly blocking acetate synthesis pathways was proposed.

Materials and methods

Strains, plasmids, primers, media, and culture conditions

Strains, plasmids, and primers are listed in Table 1. The lactate-deficient *K. pneumoniae* strain KG2 (Zhu et al. 2015), which was derived from *K. pneumoniae* strain KG (CCTCC M2014574) by deleting the *ldhA* gene, is a high 1, 3-PD producer, and it was used as the parent strain in this study.

Batch culture in 250-mL shake flask containing 50 mL of medium with 5 % inoculum for the assessment of mutants was performed at 37 °C for 24 h with shaking at 200 rpm under aerobic and anaerobic conditions. Pre-culture was cultivated in 10-mL test tube with 5 mL Luria-Bertani broth (LB) (37 °C, 18 h). The shake flask medium (pH 7.0) contained 60 g/L glycerol (or glucose), 1 g/L KCl, 2.2 g/L $KH_2PO_4{\cdot}2H_2O$, 4 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4{\cdot}7H_2O$, and 2 g/L yeast extract. To test the effect of CO_2 on bacterial growth, 1 g/L $NaHCO_3$ was added to the medium. For aerobic cultures, flasks were covered with eight layers of gauze to permit air penetration; for anaerobic cultures, the air in the flasks was replaced with nitrogen gas before cultivation, and then, the flasks were plugged with a gas-impermeable rubber stopper.

Flask-scale batch fermentation for 1,3-PD production was performed micro-aerobically at 37 °C for 24 h with shaking at 50 rpm; the medium and other procedure were the same as those for aerobic flask culture mentioned above. Fed-batch fermentation for 1,3-PD production was performed in a 5-L stirred reactor (Shanghai Bailun Biotechnology Co., Ltd., Shanghai, China) with a working volume of 2 L. The reactor was inoculated with 5 % (*v*/*v*)

Table 1 Strains, plasmids, and primers used in this study

Strain, plasmid, or primers	Relevant genotype and description	Reference or source
Strains		
K. pneumoniae KG2	Parent type, Amp^r Δ*ldhA*	Zhu et al. (2015)
K. pneumoniae KG4	Amp^r Δ*ldhA*Δ*poxB*	This study
K. pneumoniae KG5	Amp^r Δ*ldhA* Δ*poxB*Δ*pta-ackA*	This study
Plasmids		
pKD4	Kan^r, Amp^r, ori R6Kgamma, rgnB	This lab
pKD46-Tc	*Tc^r*, *repA101*(*ts*),*oriR101*, *araBp-gam-bet-exo*	Gao et al.(2014)
pCP20-Tc	*Tc^r*, *Cm^r*, *ts-rep*, [*cI857*] (*lambda*)(*ts*), *FLP*	Gao et al.(2014)
Primers		
poxB-1	5′ ATGGAGATCATGAAGCAGAC 3′	
poxB-2	5′ AAGCAGCTCCAGCCTACACATAAT CAGATGCAGGTTGCC 3′	
poxB-3	5′ AGGAGGATATTCATATGGACCCTG CGGTATCAAAGGTATA 3′	
poxB-4	5′ TTACCTGAGCCAGTTGGTT 3′	
pta-ackA-1	5′ ATGTCGAGTAAGTTAGTACTGGT 3′	
pta-ackA-2	5′ AAGCAGCTCCAGCCTACACAGCCT AACTCGGCTTCTTGT 3′	
pta-ackA-3	5′ AGGAGGATATTCATATGGACCCGG TAACACCACGTACAA 3′	
pta-ackA-4	5′ TTACTTCTGCTGCTGAGCC 3′	

of an overnight culture, and then, the bacteria were incubated under micro-aerobic conditions at 37 °C for 30 h. The aeration rate was 0.1 vvm, and the agitation speed was 140 rev/min. The initial glycerol concentration was 40 g/L, and it was maintained between 15 and 25 g/L by continuously feeding with 800 g/L of glycerol into the reactor throughout the fermentation process. The pH was maintained at 6.8 by automatically adding 50 % (*w*/*v*) KOH. The fermentation medium was the same as that reported by Cui et al. (2014).

Construction of *poxB* and *poxB-pta-ackA* mutant strains using lambda Red recombination

The *poxB* and *pta-ackA* genes were deleted by lambda Red recombination (Yamamoto et al. 2009). Gene disruption cassettes with a kanamycin-resistance marker and a flippase recognition site were amplified by two-step polymerase chain reactions from the chromosomal DNA of *K. pneumoniae* strain KG2 and the pKD4 vector, respectively, using the primers listed in Table 1. To construct the *poxB* deletion mutant, pKD46-Tc was first transformed into the KG2 strain, followed by transformation with the *poxB* disruption cassette. The recombinant strain was screened by growth on LB plates supplemented with kanamycin at 37 °C for 24 h. Finally, pCP20-Tc was transformed into the recombinant strain to remove the kanamycin-resistance gene. Cells were cultured overnight at 42 °C, diluted, and plated onto solid LB to obtain single colonies, which were further screened for loss of kanamycin and tetracycline hydrochloride resistance; the resulting *poxB* mutant strain was named KG4 (Table 1). Using the same procedure, the *pta-ackA* genes were deleted from strain KG4; the *poxB-pta-ackA* mutant strain was named KG5 (Table 1).

Analytical methods

The optical density (OD) of bacterial cultures was related to the dry cell weight by an experimentally determined calibration curve. The OD at 620 nm of the bacterial cultures was measured after appropriate dilution. 1,3-PD and 2,3-BD concentrations in the fermentation samples were quantified using gas chromatography. The analysis conditions included N_2 as the carrier gas, a detector temperature of 270 °C, and a column temperature of 120 °C. Other metabolites present in the fermentation broths were quantified using a high-performance liquid chromatography system equipped with a 2487 Dual-Wavelength Absorbance Detector (Waters Corporation, Milford, MA, USA) and a Plastisil ODS column (AQ-C18, 5 μm, 250 × 4.6 mm; Welch Material, Inc., Ellicott City, MD,

USA) at a flow rate of 0.8 mL min^{-1} and a column temperature of 65 °C. The mobile phase was 0.005 M H_2SO_4. Samples were filtered through 0.45-μm filters before analysis.

Results

Inhibitory effect of the *poxB* deletion on cell growth

Because lactate-deficient *K. pneumoniae* strains are commonly used for industrial applications, the lactate-deficient strain KG2 was used as the parent strain in our study. The growth and acetate formation of the *poxB* mutant strain KG4 and the parent strain KG2 after 24 h of flask-scale cultivation under different conditions are shown in Fig. 2. An inhibitory effect of the *poxB* deletion on bacterial growth was found. Compared with the KG2 strain, the growth of the KG4 strain significantly decreased, regardless of the carbon source used and whether the cultures were grown under anaerobic or aerobic conditions. The KG4 strain exhibited larger growth defects under anaerobic conditions (64 and 55 % of the cell concentration of the KG2 strain in glycerol- and glucose-based media, respectively), while its cell concentrations were 70 and 76 % of those of the KG2 strain in glycerol and glucose-based media, respectively, under aerobic culture conditions (Fig. 2a).

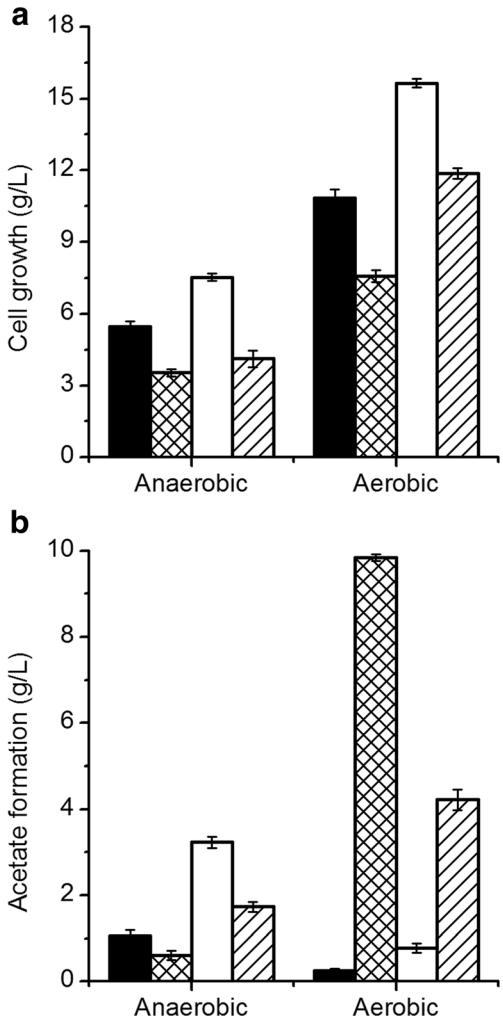

Fig. 2 Cell growth (**a**) and acetate formation (**b**) of KG2 and KG4 after 24 h of flask cultivation under anaerobic and aerobic conditions. *Black bars* KG2 in glycerol-based medium, *cross-hatched bars* KG4 in glycerol-based medium, *white bars* KG2 in glucose-based medium, *hatched bars* KG4 in glucose-based medium. *Error bars* represent the standard deviations from three independent experiments

Under anaerobic culture conditions, although the total acetate formation in strain KG4 decreased, the acetate formation per cell (g/g) was 0.17 and 0.42 in glycerol- and glucose-based media, respectively, which were almost the same as those in the KG2 strain, indicating that deleting *poxB* did not affect acetate formation. In contrast, compared with strain KG2, acetate formation in the KG4 strain was dramatically increased under aerobic conditions; meanwhile, more acetate (9.84 g/L) accumulated in a glycerol-based medium at the end of the cultivation (Fig. 2b).

In addition to acetate formation, another role of *poxB* is to generate CO_2. To assess the effect of CO_2 on the growth defect of the KG4 strain, 1 g/L of $NaHCO_3$, an efficient source of CO_2 for bacterial growth (Kozliak et al. 1995; Repaske and Clayton 1978), was added to the medium. As shown in Fig. 3, the addition of $NaHCO_3$ had no effect on the growth of the KG2 strain under all culture conditions, and it did not affect the growth of the KG4 strain under aerobic culture conditions. However, the addition of $NaHCO_3$ stimulated the anaerobic growth of strain KG4 in both glycerol- and glucose-based media, indicating that the growth defect of the KG4 strain was probable due to the shortage of CO_2 under anaerobic conditions.

Recovery of cell growth in the *poxB* mutant by deleting *pta* and *ackA*

In contrast to the adverse effects of the *poxB* deletion, deleting the other main acetate pathway, Pta-AckA, in strain KG2 had almost no effect on cell growth and the formation of metabolites, such as acetate and 1,3-PD, in all of the test conditions (data not shown). However, according to the aforementioned experiments, deleting *poxB* resulted in an extreme accumulation of acetate under aerobic culture conditions, which could be the reason for the aerobic growth defect of strain KG4. Thus, the *pta* and *ackA* genes were deleted from strain KG4. The growth and acetate formation of the resulting mutant, KG5, in a glycerol-based medium after 24 h of flask cultivation are shown in Table 2. Compared with strain KG4, acetate formation in strain KG5 dramatically decreased to 1.85 g/L (19 % of that of the KG4 strain) under aerobic conditions (Table 2). As a result of the decreased accumulation of acetate, the concentration of KG5 cells significantly increased at the

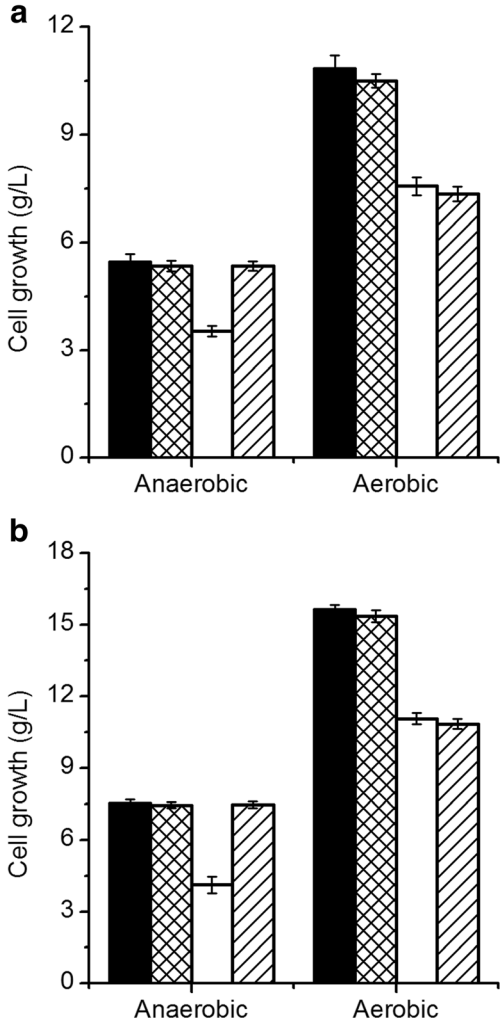

Fig. 3 The effect of $NaHCO_3$ on cell growth of KG2 and KG4 at the end of flask cultivation under anaerobic and aerobic conditions in glycerol-based medium (**a**) or glucose-based medium (**b**). *Black bars* KG2 without $NaHCO_3$, *white bars* KG4 without $NaHCO_3$, *cross-hatched bars* KG2 with $NaHCO_3$, *hatched bars* KG4 with $NaHCO_3$. *Error bars* represent the standard deviations from three independent experiments

end of aerobic cultivation, almost reaching the level of the parent strain KG2 (Table 2). More interestingly, it was shown that deleting the *pta-ackA* genes alleviated the growth defect of the *poxB* mutant, even under aerobic condition. At the end of anaerobic cultivation, the cell concentration of KG5 was the same as that of KG2 (Table 2). Such recovery of cell growth in the KG5 strain under both aerobic and anaerobic conditions was confirmed in a glucose-based medium (data not shown).

Although the growth of strain KG5 was almost the same as that of the KG2 strain in aerobic and anaerobic cultures, the varieties of metabolites formed, especially those involved in the TCA cycle, differed (Table 2). The levels of α-ketoglutarate and succinate in strain KG5 were only 62 and 41 %, respectively, of those in strain KG2 at the end of aerobic cultivation. In contrast, the levels of α-ketoglutarate and succinate substantially increased in strain KG5 at the end of anaerobic cultivation, as they were approximately 211 and 53 %, respectively, greater than those in strain KG2. Interestingly, the highest level of 1,3-PD production (20.8 g/L) at the end of anaerobic cultivation was found in strain KG5, which was 15 % greater than that in strain KG2 (Table 2).

Pyruvate accumulation is usually reported to be the reason for the growth defects in Pta-AckA-deficient *E. coli* strains (Chang et al. 1999; Liao et al. 1996). Based on our experiments, deleting *poxB* resulted in a high accumulation of pyruvate in strain KG4 under anaerobic culture condition, while strain KG5 did not accumulate high levels of pyruvate at the end of anaerobic cultivation (Table 2). Because of the importance of the pyruvate node in metabolism, variations in the level of pyruvate in strain KG5 during flask cultures were further analyzed. As shown in Fig. 4a, pyruvate accumulation in strain KG5 was found during the early stage of flask cultivation. The peak values of pyruvate occurred after approximately 6 h and reached 0.72 g/L (aerobic conditions) and 0.31 g/L (anaerobic conditions). The growth of strain KG5 during flask cultivation is also shown in Fig. 5. Compared with the KG2 strain, the growth of strain KG5 was retarded during the early stage of flask cultivation. The growth

Table 2 Cell growth and production of KG2, KG4, and KG5 in glycerol-based medium under aerobic and anaerobic conditions at 24 h in shake flask

	Aerobic			Anaerobic		
	KG2	KG4	KG5	KG2	KG4	KG5
Cell growth (g/L)	10.8 ± 0.4	7.57 ± 0.25	10.2 ± 0.2	5.46 ± 0.22	3.53 ± 0.15	5.11 ± 0.13
1,3-PD (g/L)	10.0 ± 0.3	6.12 ± 0.15	11.3 ± 0.2	18.1 ± 0.4	13.7 ± 0.2	20.8 ± 0.4
2,3-BD (g/L)	6.25 ± 0.45	3.64 ± 0.26	6.81 ± 0.28	4.68 ± 0.36	2.28 ± 0.24	4.81 ± 0.35
Pyruvate (g/L)	0.04 ± 0.01	0.08 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	4.89 ± 0.05	0.02 ± 0.01
Acetate (g/L)	0.25 ± 0.05	9.84 ± 0.08	1.85 ± 0.05	1.05 ± 0.14	0.61 ± 0.11	0.36 ± 0.05
Succinate (g/L)	1.64 ± 0.04	0.42 ± 0.08	0.67 ± 0.06	2.71 ± 0.08	2.24 ± 0.05	4.14 ± 0.08
α-Ketoglutarate (g/L)	0.37 ± 0.04	0.13 ± 0.04	0.23 ± 0.06	0.09 ± 0.02	0.04 ± 0.01	0.28 ± 0.05

All date are expressed as the mean ± SD of three independent experiments

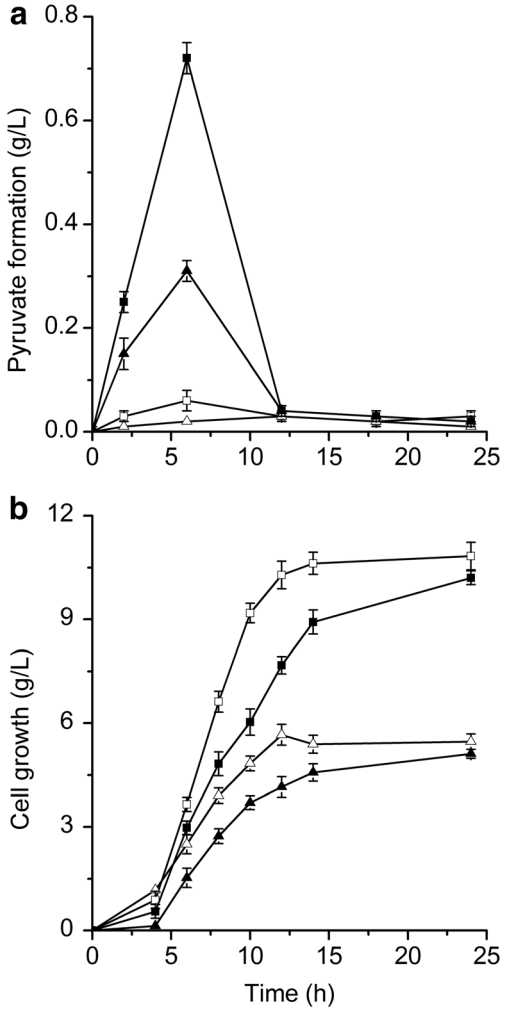

Fig. 4 Pyruvate formation (**a**) and cell growth (**b**) of KG2 (*open symbol*) and KG5 (*solid symbol*) during the shaker flask cultivation. Aerobic (*square*) and anaerobic (*triangle*). Date points are averages of three identical experiments

retardation was greater under aerobic conditions. At 10 h of cultivation, the cell concentration of strain KG5 was 66 % of that of strain KG2, while under anaerobic conditions, it reached 77 % of that of the KG2 strain (Fig. 4b).

1,3-PD production by the *poxB-pta-ackA* mutant KG5 during fed-batch fermentation

Compared with anaerobic fermentation, micro-aerobic fermentation for 1,3-PD production by *K. pneumoniae* is commonly used recently due to its high process productivity and convenient industrial application (Chen et al. 2003; Durgapal et al. 2014). As expected, the strain of KG5 also showed the high ability for 1,3-PD production under micro-aerobic condition in a flask batch process (Table 3). To confirm the ability of strain KG5 to produce large amounts of 1,3-PD, fed-batch fermentations under micro-aerobic conditions were

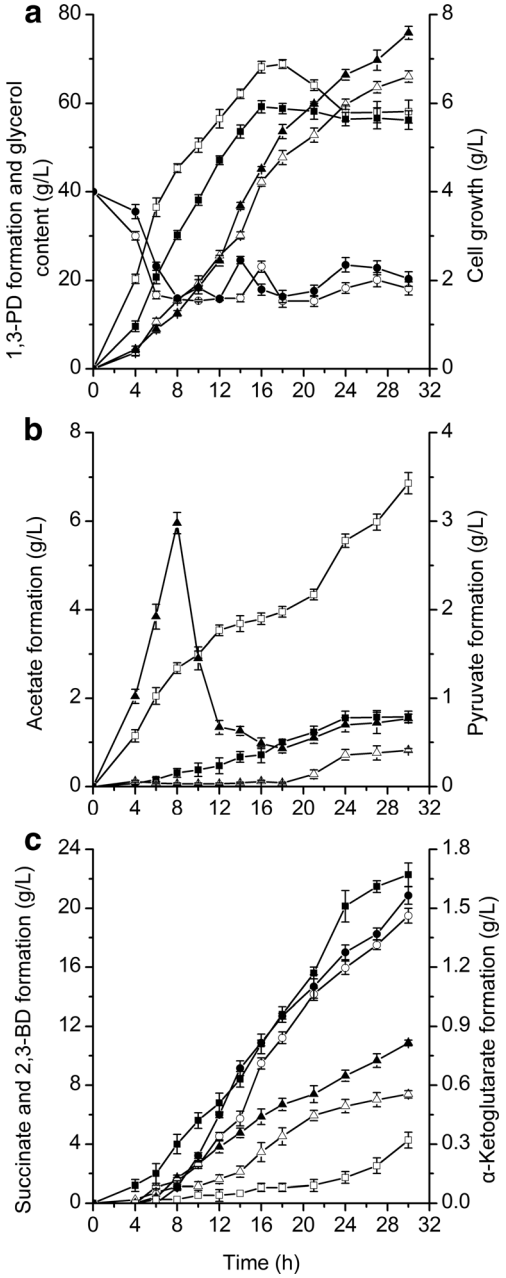

Fig. 5 Cell growth and metabolites formation in KG2 (*open symbol*) and KG5 (*solid symbol*) during the fed-batch fermentation process under micro-aerobic condition. **a** 1,3-PD (*triangle*), glycerol (*circle*), and cell growth (*square*); **b** acetate (*square*) and pyruvate (*triangle*); **c** α-ketoglutarate (*square*), 2,3-BD (*circle*), and succinate (*triangle*). Date points are averages of three identical experiments

performed in a 5-L bioreactor. The detailed fermentation results are shown in Fig. 5 and Table 3. During the fed-batch fermentation, the cell concentration increased in the early stage (0–16 h) and decreased in the later stage (16–30 h). Compared with the KG2 strain, the growth of strain KG5 was lower in the early stage, although a small decrease in the cell concentration was found in the later stage. In contrast to growth, a high level of 1,3-PD

Table 3 Fermentation results of KG2 and KG5 under micro-aerobic condition in flask batch and bioreactor fed-batch processes

Processes	Strains	1,3-PD (g/L)	2,3-BD (g/L)	Acetate (g/L)	1,3-PD productivity (g/L h)	1,3-PD yield (mol/mol)	Glycerol consumed (g/L)
24 h of flask batch	KG2	19.4 ± 0.5	5.48 ± 0.26	1.56 ± 0.15	0.81 ± 0.03	0.40 ± 0.02	58.4 ± 1.1
	KG5	22.5 ± 0.6	5.65 ± 0.25	0.48 ± 0.06	0.94 ± 0.02	0.47 ± 0.02	57.8 ± 1.2
30 h of 5-L bioreactor fed batch	KG2	66.9 ± 1.3	19.5 ± 0.5	6.86 ± 0.24	2.23 ± 0.04	0.57 ± 0.02	141.3 ± 1.3
	KG5	76.8 ± 1.5	20.8 ± 0.6	1.58 ± 0.13	2.56 ± 0.05	0.66 ± 0.03	140.1 ± 1.2

All date are expressed as the mean ± SD of three independent experiments

production was observed in strain KG5, especially in the later stage of fermentation (Fig. 5a). At the end of the fed-batch fermentation, 1,3-PD production by strain KG5 reached 76.8 g/L, which was 15 % greater than that of strain KG2.

Deleting the *poxB*, *pta*, and *ackA* genes resulted in a significant decrease in acetate formation during fed-batch fermentation. Acetate production by strain KG5 was 1.58 g/L, only 23 % of that of strain KG2 at the end of the fed-batch fermentation. In contrast with strain KG2, greater accumulations of α-ketoglutarate and succinate were observed in strain KG5 during fed-batch fermentation, and a high accumulation of pyruvate during the early stage of fermentation was also found. As shown in Table 3, strain KG5 completely consumed 140.1 g/L of glycerol within 30 h of fed-batch fermentation, which was less than that consumed by strain KG2 (141.3 g/L). Meanwhile, the formation of the dominant by-product, 2,3-BD, was almost unchanged; thus, the total yield of 1,3-PD from glycerol by strain KG5 in fed-batch fermentation reached 0.66 (mol/mol), a 16 % increase compared with strain KG2 (Table 3).

Discussion

Acetate, which is highly toxic, is a major by-product that is excreted during the formation of 1,3-PD from glycerol in *K. pneumoniae* (Celinska 2010; Zeng et al. 1994). Although a low level of acetate seems to have a positive effect on 1,3-PD fermentation (Grahame et al. 2013; Lu et al. 2013), acetate overflow could lead to inhibition of cell growth and a decrease in 1,3-PD production (Cui et al. 2014; He et al. 2013; Szymanowska-Powalowska and Kubiak 2015). In this study, the possibility of improving 1,3-PD production by directly blocking acetate synthesis pathways was investigated. Our results indicated that, in addition to decreasing acetate formation, deleting the *poxB*, *pta*, and *ackA* genes significantly improved 1,3-PD production in *K. pneumoniae*.

PoxB catalyzes the oxidative decarboxylation of pyruvate to acetate. In *E. coli*, pyruvate is mainly converted into acetyl-CoA by the dehydrogenase complex (PDHC) and/or pyruvate formate-lyase (PFL), so PoxB generally has been regarded as non-essential (Chang et al. 1994; Grabau and Cronan 1984). Although previous studies have elucidated some functions of PoxB, deleting *poxB* had almost no effect on aerobic growth and acetate formation in *E. coli* (Chang and Cronan 1983; Phue et al. 2010). In our experiments, an extreme growth defect occurred in the *poxB* mutant, indicating that, in contrast to *E. coli*, PoxB plays a more important role in *K. pneumoniae*. As the recycling of PoxB-generated acetate to acetyl-CoA via ACS requires additional energy, the involvement of PoxB in pyruvate oxidation seems wasteful. However, PoxB was actually confirmed to contribute to aerobic growth in *E. coli* (Abdel-Hamid et al. 2001). Thus, the sizeable acetate flux generated by PoxB could be an adaptation to ensure good growth in *K. pneumoniae*.

Although the deletion of *poxB* inhibited growth in both aerobic and anaerobic culture conditions, the reasons for the growth defects differ. Under aerobic conditions, the extra carbon flux obtained by blocking PoxB should directly flow to acetyl-CoA via PDHC and/or PFL. Meanwhile, the TCA cycle and acetate assimilation were reported to be suppressed in a *poxB* mutant (Kumari et al. 2000; Li et al. 2006; Martinez-Gomez et al. 2012; Phue and Shiloach 2004); thus, a high concentration of acetate accumulated in the *poxB* mutant, probably through the Pta-AckA pathway. Compared with the glucose-based medium, greater acetate accumulation in the *poxB* mutant correlated with a more severe aerobic growth defect in the glycerol-based medium. Consequently, the accumulation of acetate in aerobic cultures could explain the growth defect of the *poxB* mutant. Carbon-dependent accumulation of acetate in the *poxB* mutant may be caused by regulatory differences in acetate assimilation (Wolfe 2005). The suppression of the TCA cycle in an aerobic culture of the *poxB* mutant was confirmed, as evidenced by the decrease in metabolites involved in the TCA cycle, such as α-ketoglutarate and succinate (Table 2). Additionally, under anaerobic conditions, CO_2 is important for bacterial growth (Dharmadi et al. 2006; Merlin et al. 2003; Tran et al. 2014), and metabolic pathways, such as the PoxB pathway, should be involved in producing an endogenous supply of CO_2 because of the lack of TCA. Thus, the serious growth defect of the *poxB* mutant implies that an appreciable amount of PoxB-generated CO_2 is required for bacterial growth. The alleviation

of the anaerobic growth defect of the *poxB* mutant by the addition of $NaHCO_3$ (an external CO_2 donor) to the medium strongly supports the above assumption (Fig. 3). It was reported that the requirement for CO_2 for bacterial growth mainly depended on PDHC in anaerobically grown *E. coli* (Murarka et al. 2010). Here, it was shown that PoxB affected the generation of CO_2 during the anaerobic growth of *K. pneumoniae*. Based on our data, compared with aerobic growth, deleting *poxB* resulted in a great accumulation of pyruvate and a more severe inhibition of anaerobic growth of *K. pneumoniae* (Table 2 and Fig. 2).

The Pta-AckA pathway is considered to be the primary acetate synthesis pathway in *E. coli* (De Mey et al. 2007; Merlin et al. 2003). In our study, the deletion of *pta-ackA*, unlike the deletion of *poxB*, had no effect on bacterial growth and metabolite formation in *K. pneumoniae*. However, the growth and metabolic characteristics of the *poxB-pta-ackA* mutant significantly differed from those of the *poxB* mutant. Under aerobic conditions, a large decrease in acetate formation occurred in the *poxB-pta-ackA* mutant, which strongly suggests that Pta-AckA also plays an important role in acetate formation, although the Pta-AckA pathway is dispensable in a *K. pneumoniae* strain that expresses PoxB. Accompanying the decrease in acetate formation, the aerobic growth of the *poxB-pta-ackA* mutant was better than that of the *poxB* mutant. The cell concentration of the *poxB-pta-ackA* mutant at the end of flask cultivation almost recovered to the same level of the parent strain. Thus, this further confirmed that the high accumulation of acetate was responsible for the aerobic growth defect of the *poxB* mutant. More interestingly, the alleviation of the growth defect of the *poxB* mutant by deleting the *pta-ackA* even occurred under aerobic conditions. This could mean that, in addition to the decrease in acetate formation in aerobic conditions, deleting *pta-ackA* could also overcome the shortage of CO_2 in the *poxB* mutant under anaerobic conditions. According to our data, the increase in metabolites involved in the TCA cycle (α-ketoglutarate and succinate) clearly suggests that carbon flux could be forced to the TCA cycle in the *poxB-pta-ackA* mutant under anaerobic conditions, and an increased flux of the TCA cycle can supply sufficient CO_2 for the anaerobic growth of *K. pneumoniae*.

Increasing the carbon flux to the TCA cycle could generate extra dihydronicotinamide adenine dinucleotide (NADH), which is helpful for 1,3-PD production. Further studies indicated that the cell growth rate of *poxB-pta-ackA* mutant was still little lower than that of parent strain, and accumulation of pyruvate was also found in the early stage of cultivation, which was observed in Pta-AckA deficient *E. coli* strains (Chang et al. 1999; Liao et al. 1996). Nevertheless, the *poxB-pta-ackA* mutant showed an excellent ability to produce 1,3-PD under both anaerobic and micro-aerobic conditions. Based on our data, deleting *poxB-pta-ackA* severely decreased acetate formation and increased the carbon flux to the TCA cycle, while there was no effect on 2,3-BD formation. As a result, the 1,3-PD concentration and yield reached 76.8 g/L and 0.66 (mol/mol), respectively, after 30 h of fed-batch fermentation, which were 15 and 16 %, respectively, greater than those of the parent strain.

In summary, a *K. pneumoniae poxB* mutant exhibited an extreme growth defect. The growth defect of the *poxB* mutant was alleviated by deleting *pta-ackA*, probably due to the decrease in acetate formation under aerobic conditions and the improvement in the CO_2 supply under anaerobic conditions. According to our data, deleting *poxB*, *pta*, and *ackA* in *K. pneumoniae* severely decreased acetate formation and increased carbon flux to the TCA cycle, which was improved 1,3-PD production.

Acknowledgments This work was supported by the National Natural Science Foundation of China under Grant No. 31271862.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Human and animal studies This article does not contain any studies with human participants or animals performed by any of the author.

References

Abdel-Hamid AM, Attwood MM, Guest JR (2001) Pyruvate oxidase contributes to the aerobic growth efficiency of *Escherichia coli*. Microbiology 147:1483–1498

Ashok S, Raj SM, Rathnasingh C, Park S (2011) Development of recombinant *Klebsiella pneumoniae* delta dhaT strain for the co-production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol. Appl Microbiol Biotechnol 90:1253–1265

Celinska E (2010) Debottlenecking the 1,3-propanediol pathway by metabolic engineering. Biotechnol Adv 28:519–530

Celinska E (2012) *Klebsiella* spp as a 1, 3-propanediol producer: the metabolic engineering approach. Crit Rev Biotechnol 32:274–288

Chang DE, Shin S, Rhee JS, Pan JG (1999) Acetate metabolism in a *pta* mutant of *Escherichia coli* W3110: importance of maintaining acetyl coenzyme A flux for growth and survival. J Bacteriol 181:6656–6663

Chang YY, Cronan Jr JE (1983) Genetic and biochemical analyses of *Escherichia coli* strains having a mutation in the structural gene (poxB) for pyruvate oxidase. J Bacteriol 154:756–762

Chang YY, Wang AY, Cronan Jr JE (1994) Expression of *Escherichia coli* pyruvate oxidase (PoxB) depends on the sigma factor encoded by the rpoS(katF) gene. Mol Microbiol 11:1019–1028

Chen X, Zhang DJ, Qi WT, Gao SJ, Xiu ZL, Xu P (2003) Microbial fed-batch production of 1,3-propanediol by *Klebsiella pneumoniae* under micro-aerobic conditions. Appl Microbiol Biotechnol 63:143–146

Cui YL, Zhou JJ, Gao LR, Zhu CQ, Jiang X, Fu SL, Gong H (2014) Utilization of excess NADH in 2,3-butanediol-deficient *Klebsiella pneumoniae* for 1,3-propanediol production. J Appl Microbiol 117: 690–698

De Mey M, De Maeseneire S, Soetaert W, Vandamme E (2007) Minimizing acetate formation in *E. coli* fermentations. J Ind Microbiol Biotechnol 34:689–700

Dharmadi Y, Murarka A, Gonzalez R (2006) Anaerobic fermentation of glycerol by *Escherichia coli*: a new platform for metabolic engineering. Biotechnol Bioeng 94:821–829

Dittrich CR, Vadali RV, Bennett GN, San KY (2005) Redistribution of metabolic fluxes in the central aerobic metabolic pathway of *E. coli* mutant strains with deletion of the *ackA-pta* and *poxB* pathways for the synthesis of isoamyl acetate. Biotechnol Prog 21:627–631

Durgapal M, Kumar V, Yang TH, Lee HJ, Seung D, Park S (2014) Production of 1,3-propanediol from glycerol using the newly isolated *Klebsiella pneumoniae* J2B. Bioresour Technol 159:223–231

Gao L, Jiang X, Fu S, Gong H (2014) In silico identification of potential virulence genes in 1,3-propanediol producer *Klebsiella pneumoniae*. J Biotechnol 189:9–14

Grabau C, Cronan Jr JE (1984) Molecular cloning of the gene (poxB) encoding the pyruvate oxidase of *Escherichia coli*, a lipid-activated enzyme. J Bacteriol 160:1088–1092

Grahame DA, Kang TS, Khan NH, Tanaka T (2013) Alkaline conditions stimulate the production of 1,3-propanediol in *Lactobacillus panis* PM1 through shifting metabolic pathways. World J Microbiol Biotechnol 29:1207–1215

He L, Zhao X, Cheng K, Sun Y, Liu D (2013) Kinetic modeling of fermentative production of 1, 3-propanediol by *Klebsiella pneumoniae* HR526 with consideration of multiple product inhibitions. Appl Biochem Biotechnol 169:312–326

Huang Y, Li Z, Shimizu K, Ye Q (2012) Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol by a recombinant strain of *Klebsiella pneumoniae*. Bioresour Technol 103: 351–359

Khan NH, Kang TS, Grahame DA, Haakensen MC, Ratanapariyanuch K, Reaney MJ, Korber DR, Tanaka T (2013) Isolation and characterization of novel 1,3-propanediol-producing *Lactobacillus panis* PM1 from bioethanol thin stillage. Appl Microbiol Biotechnol 97:417–428

Kozliak EI, Fuchs JA, Guilloton MB, Anderson PM (1995) Role of bicarbonate/CO_2 in the inhibition of *Escherichia coli* growth by cyanate. J Bacteriol 177:3213–3219

Kumar V, Sankaranarayanan M, Durgapal M, Zhou S, Ko Y, Ashok S, Sarkar R, Park S (2013) Simultaneous production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol using resting cells of the lactate dehydrogenase-deficient recombinant *Klebsiella pneumoniae* overexpressing an aldehyde dehydrogenase. Bioresour Technol 135:555–563

Kumar V, Sankaranarayanan M, Jae KE, Durgapal M, Ashok S, Ko Y, Sarkar R, Park S (2012) Co-production of 3-hydroxypropionic acid and 1,3-propanediol from glycerol using resting cells of recombinant *Klebsiella pneumoniae* J2B strain overexpressing aldehyde dehydrogenase. Appl Microbiol Biotechnol 96:373–383

Kumari S, Simel EJ, Wolfe AJ (2000) sigma(70) is the principal sigma factor responsible for transcription of *acs*, which encodes acetyl coenzyme A synthetase in *Escherichia coli*. J Bacteriol 182:551–554

Lee SM, Hong WK, Heo SY, Park JM, Jung YR, Oh BR, Joe MH, Seo JW, Kim CH (2014) Enhancement of 1,3-propanediol production by expression of pyruvate decarboxylase and aldehyde dehydrogenase from Zymomonas mobilis in the acetolactate-synthase-deficient mutant of *Klebsiella pneumoniae*. J Ind Microbiol Biotechnol 41:1259–1266

Li M, Yao S, Shimizu K (2006) Effect of poxB gene knockout on metabolism in *Escherichia coli* based on growth characteristics and enzyme activities. World J Microbiol Biotechnol 23:573–580

Li M, Zhang X, Agrawal A, San KY (2012) Effect of acetate formation pathway and long chain fatty acid CoA-ligase on the free fatty acid production in *E. coli* expressing acy-ACP thioesterase from Ricinus communis. Metab Eng 14:380–387

Liao JC, Hou SY, Chao YP (1996) Pathway analysis, engineering, and physiological considerations for redirecting central metabolism. Biotechnol Bioeng 52:129–140

Lu S, Han Y, Duan X, Luo F, Zhu L, Li S, Huang H (2013) Cell morphology variations of *Klebsiella pneumoniae* induced by acetate stress using biomimetic vesicle assay. Appl Biochem Biotechnol 171:731–743

Martinez-Gomez K, Flores N, Castaneda HM, Martinez-Batallar G, Hernandez-Chavez G, Ramirez OT, Gosset G, Encarnacion S, Bolivar F (2012) New insights into *Escherichia coli* metabolism: carbon scavenging, acetate metabolism and carbon recycling responses during growth on glycerol. Microb Cell Factories 11:46

Merlin C, Masters M, McAteer S, Coulson A (2003) Why is carbonic anhydrase essential to *Escherichia coli*? J Bacteriol 185:6415–6424

Murarka A, Clomburg JM, Moran S, Shanks JV, Gonzalez R (2010) Metabolic analysis of wild-type *Escherichia coli* and a pyruvate dehydrogenase complex (PDHC)-deficient derivative reveals the role of PDHC in the fermentative metabolism of glucose. J Biol Chem 285:31548–31558

Peebo K, Valgepea K, Nahku R, Riis G, Oun M, Adamberg K, Vilu R (2014) Coordinated activation of PTA-ACS and TCA cycles strongly reduces overflow metabolism of acetate in *Escherichia coli*. Appl Microbiol Biotechnol 98:5131–5143

Petrov K, Petrova P (2009) High production of 2,3-butanediol from glycerol by *Klebsiella pneumoniae* G31. Appl Microbiol Biotechnol 84: 659–665

Phue JN, Lee SJ, Kaufman JB, Negrete A, Shiloach J (2010) Acetate accumulation through alternative metabolic pathways in *ackA*$^-$ *pta*$^-$ *poxB*$^-$ triple mutant in *E. coli* B (BL21). Biotechnol Lett 32: 1897–1903

Phue JN, Shiloach J (2004) Transcription levels of key metabolic genes are the cause for different glucose utilization pathways in *E. coli* B (BL21) and *E. coli* K (JM109). J Biotechnol 109:21–30

Repaske R, Clayton MA (1978) Control of *Escherichia coli* growth by CO_2. J Bacteriol 135:1162–1164

Seo MY, Seo JW, Heo SY, Baek JO, Rairakhwada D, Oh BR, Seo PS, Choi MH, Kim CH (2009) Elimination of by-product formation during production of 1,3-propanediol in *Klebsiella pneumoniae* by inactivation of glycerol oxidative pathway. Appl Microbiol Biotechnol 84:527–534

Skraly FA, Lytle BL, Cameron DC (1998) Construction and characterization of a 1,3-propanediol operon. Appl Environ Microbiol 64:98–105

Szymanowska-Powalowska D, Bialas W (2014) Scale-up of anaerobic 1, 3-propanediol production by *Clostridium butyricum* DSP1 from crude glycerol. BMC Microbiol 14:45

Szymanowska-Powalowska D, Kubiak P (2015) Effect of 1,3-propanediol, organic acids, and ethanol on growth and metabolism of *Clostridium butyricum* DSP1. Appl Microbiol Biotechnol 99: 3179–3189

Tang X, Tan Y, Zhu H, Zhao K, Shen W (2009) Microbial conversion of glycerol to 1,3-propanediol by an engineered strain of *Escherichia coli*. Appl Environ Microbiol 75:1628–1634

Tran KT, Maeda T, Wood TK (2014) Metabolic engineering of *Escherichia coli* to enhance hydrogen production from glycerol. Appl Microbiol Biotechnol 98:4757–4770

Wolfe AJ (2005) The acetate switch. Microbiol Mol Biol Rev 69:12–50

Xu YZ, Guo NN, Zheng ZM, Ou XJ, Liu HJ, Liu DH (2009) Metabolism in 1,3-propanediol fed-batch fermentation by a D-lactate deficient mutant of *Klebsiella pneumoniae*. Biotechnol Bioeng 104:965–972

Yamamoto S, Izumiya H, Morita M, Arakawa E, Watanabe H (2009) Application of lambda Red recombination system to Vibrio cholerae genetics: simple methods for inactivation and modification of chromosomal genes. Gene 438:57–64

Yen HW, Li FT, Chang JS (2014) The effects of dissolved oxygen level on the distribution of 1,3-propanediol and 2,3-butanediol produced from glycerol by an isolated indigenous *Klebsiella* sp. Ana-WS5. Bioresour Technol 153:374–378

Zeng AP, Ross A, Biebl H, Tag C, Gunzel B, Deckwer WD (1994) Multiple product inhibition and growth modeling of Clostridium butyricum and *Klebsiella pneumoniae* in glycerol fermentation. Biotechnol Bioeng 44:902–911

Zheng ZM, Xu YZ, Liu HJ, Guo NN, Cai ZZ, Liu DH (2008) Physiologic mechanisms of sequential products synthesis in 1,3-propanediol fed-batch fermentation by *Klebsiella pneumoniae*. Biotechnol Bioeng 100:923–932

Zhu C, Jiang X, Zhang Y, Lin J, Fu S, Gong H (2015) Improvement of 1, 3-propanediol production in *Klebsiella pneumoniae* by moderate expression of *puuC* (encoding an aldehyde dehydrogenase). Biotechnol Lett 37:1783–1790

Zhuge B, Zhang C, Fang HY, Zhuge JA, Permaul K (2010) Expression of 1,3-propanediol oxidoreductase and its isoenzyme in *Klebsiella pneumoniae* for bioconversion of glycerol into 1,3-propanediol. Appl Microbiol Biotechnol 87:2177–2184