Metabolic engineering of *Escherichia coli* to produce gamma-aminobutyric acid using xylose

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Abstract Biomass-derived xylose is an economically interesting substrate for the sustainable microbial production of value-added compounds. *Escherichia coli* could barely use xylose to directly produce gamma-aminobutyric acid. In this study, *E. coli* strains that could directly produce gamma-aminobutyric acid were developed through the deletion of eight genes *sucA*, *puuE*, *gabT*, *gabP*, *xylA*, *xylB*, *waaC*, and *waaF*, and the overexpression of two *E. coli* genes *gadB* and *gdhA*, as well as five *Caulobacter crescent* genes *CcxylA*, *CcxylB*, *CcxylC*, *CcxylD*, and *CcxylX*. Both *E. coli* strains W3110 and JM109 could directly produce gamma-aminobutyric acid from xylose after either overexpression of the seven genes or deletion of the eight genes. Overexpression of the seven genes of in the multiple deletion mutants further increased gamma-aminobutyric acid production. Among the 28 recombinant *E. coli* strains constructed in this study, the highest gamma-aminobutyric acid was produced by JWZ08/pWZt7-g3/pWZt7-xyl. JWZ08/pWZt7-g3/pWZt7-xyl could produce 3.95 g/L gamma-aminobutyric acid in flask cultivation, using xylose as the sole carbon source.

Keywords Gamma-aminobutyric acid · GABA · Xylose · L-glutamic acid · Glu · *Escherichia coli*

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Introduction

Gamma-aminobutyric acid (GABA), a four-carbon nonprotein amino acid, is the main inhibitory neurotransmitter in human cortex (Wong et al. 2003). It has a potential use as food additive or dietary supplement for its physiological functions, such as blood pressure decrease, anxiety, and even diabetes inhibition (Adeghate and Ponery 2002; Boonstra et al. 2015). In addition, GABA can be also used as the precursor for synthesizing the biodegradable polymer nylon 4 (Park et al. 2013).

Biosynthesis of GABA has been studied over the past decade. Glutamate decarboxylase (Gad), the key enzyme for the GABA production in microorganisms, catalyzes L-glutamic acid (Glu) or monosodium glutamate (MSG) decarboxylation to form GABA. The mostly used bacteria for GABA production are lactic acid bacteria and *Escherichia coli*. Lactic acid bacteria are generally regarded as safe, therefore, the GABA-producing lactic acid bacteria can be directly used in some functional food. Many GABA-producing lactic acid bacteria have been isolated, such as *Lactobacillus brevis* (Huang et al. 2007; Kim et al. 2009; Zhang et al. 2012), *Lactobacillus paracasei* (Komatsuzaki et al. 2005), *Lactobacillus buchneri* (Zhao et al. 2015), *Lactobacillus plantarum* (Siragusa et al. 2007), *Lactococcus lactis* (Lacroix et al. 2013; Lu et al. 2008), and *Enterococcus avium* (Tamura et al. 2010). GABA productions lactic acid bacteria can be enhanced by condition optimization. For example, GABA production in *L. brevis* NCL912 increased sixfold by optimizing the media and controlling pH (Li et al. 2010a; Li et al. 2010b). When Gad was overexpressed in *L. plantarum* Taj-Apis362, only 11 mM (1.1 g/L) GABA was produced from 0.5 M Glu (Tajabadi et al. 2015). Successful genetic engineering in lactic acid bacteria for GABA production has not been reported.

GABA production in *E. coli* can be efficiently improved by genetic engineering. Though wild *E. coli* does not produce GABA from MSG or Glu, 5.07 g/L GABA was produced from 10 g/L of MSG in *E. coli* XL1-Blue overexpressing *Pyrococcus horikoshii* Gad (Le Vo et al. 2014). *E. coli* cells overexpressing Gad can also be used as whole cell biocatalyst to repeatedly convert Glu or MSG to GABA in buffer (Yamano et al. 2012). GABA production from MSG can be dramatically increased in *E. coli* when GadB was secreted with the help of signal peptide TorA (Zhao et al. 2016).

Since Glu and MSG can be mass-produced by *Corynebacterium glutamicum*, they are often used as the precursor for GABA production. However, from the industrial and economical point of view, production of GABA directly from glucose is attractive. In *C. glutamicum* ATCC13032, 0.88 g/L GABA was produced from glucose after 36-h flask cultivation, by overexpressing *gadB2* from *L. brevis* Lb85 (Shi and Li 2011); 26.32 g/L GABA was produced from glucose after 60-h fed-batch fermentation, by overexpressing *gadB1* and *gadB2* from *L. brevis* Lb85 (Shi et al. 2013). GABA (70.6 g/L) was produced in *C. glutamicum* G01 overexpressing Gad from *L. plantarum* CCTCC M209102 and blocking by-product pools of arginine, proline, and lysine, after 70-h two-stage pH control fermentation (Zhang et al. 2014). Wild *E. coli* produces neither glutamate nor GABA, but GABA-producing *E. coli* can be constructed by genetic engineering. For example, by co-overexpressing isocitrate dehydrogenase, glutamate synthase, and GadB, and introducing a synthetic protein scaffold to fix the three enzymes in *E. coli* XL1-Blue △*ackA* △*gabT*, 1.3 g/L GABA was produced from 10 g/L glucose (Pham et al. 2015). GABA can be purified by cation exchange column and crystallized by adding ethanol (Chen et al. 2016; Gao et al. 2013; Seungwoon et al. 2013).

Xylose is a major component of hemicellulose, the renewable low-cost resource. Its abundance and relative ease of extraction from biomass make it an attractive source of fermentable sugar for various productions (Aristidou and Penttilä 2000). But xylose has not been used to produce GABA in *E. coli*. *E. coli* metabolizes xylose via the isomerase pathway (Fig. 1). Xylose isomerase encoded by *xylA* converts xylose to xylulose, which is phosphorylated to lyxulose-5-phosphate by xylulokinase encoded by *xylB*. Transketolase catalyzes lyxulose-5-phosphate to glyceraldehyde-3-phosphate, the key precursor in Embden-Meyerhof-Parnas (EMP) pathway. In EMP pathway, glyceraldehyde-3-phosphate is gradually converted to phosphoenolpyruvate by enzymes glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, and enolase. Then, pyruvate kinase removes the phosphate group of phosphoenolpyruvate to yield pyruvate. Phosphoenolpyruvate and pyruvate enters TCA cycle through oxaloacetate and citrate, respectively. In TCA cycle, α-ketoglutarate is synthesized by enzymes citrate synthase, aconitase, and isocitrate

dehydrogenase. At the end, glutamate dehydrogenase encoded by *gdhA* catalyzes α-ketoglutarate to Glu, which is decarboxylated to form GABA. In addition to the isomerase pathway, Weimberg pathway represents a promising alternative for xylose assimilation (Fig. 1). Weimberg pathway is found in *Pseudomonas fragi* (Weimberg 1961), *Haloferax volcanii* (Johnsen et al. 2009), and *Caulobacter crescentus* (Stephens et al. 2007) and can metabolize xylose to α-ketoglutarate in a five-step oxidative process. In Weimberg pathway, xylose dehydrogenase encoded by *CcxylB* initiates the oxidation of xylose to xylonolactone, and then xylonolactonase encoded by *CcxylC* opens the lactone ring to form xylonate which is dehydrated twice in two successive reactions catalyzed by xylonate dehydratase encoded by *CcxylD* and 2-keto-3-deoxyxylonate dehydratase encoded by *CcxylX*, respectively. The α-ketoglutarate semialdehyde is then oxidized by α-ketoglutarate semialdehyde dehydrogenase encoded by *CcxylA*, forming α-ketoglutarate (Stephens et al. 2007). Weimberg pathway does not exist in *E. coli*. Comparing with the isomerase pathway, Weimberg pathway not only offers a more direct conversion of xylose to α-ketoglutarate but also lowers the carbon loss in the form of CO_2 (Radek et al. 2014).

In this study, Weimberg pathway was introduced into *E. coli* strains JM109 and W3110. GABA-producing *E. coli* strains were developed through deletion of eight genes *sucA*, *puuE*, *gabT*, *gabP*, *xylA*, *xylB*, *waaC*, and *waaF*, and overexpression of two *E. coli* genes *gadB* and *gdhA*, as well as five *Caulobacter crescent* genes *CcxylA*, *CcxylB*, *CcxylC*, *CcxylD*, and *CcxylX*. The final strain JWZ08/pWZt7-g3/pWZt7-xyl could produce 3.95 g/L GABA in flask cultivation, using xylose as the sole carbon source.

Materials and methods

Strains, media, and growth conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37 °C in LB medium (10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl). Ampicillin (100 μg/mL), kanamycin (30 μg/mL), and chloramphenicol (30 μg/mL) were added when necessary. *C. glutamicum* ATCC13869 was grown at 30 °C in LBG medium (LB medium supplemented with 5 g/L glucose). *C. crescentus* NA1000 was grown at 30 °C in PYE medium (2 g/L peptone, 1 g/L yeast extract, and 0.2 g/L $MgSO_4 \cdot 7H_2O$).

For flask fermentation, a loop of *E. coli* cell colonies was inoculated into 20-mL LB medium in a 250-mL flask. One-milliliter overnight seed culture was diluted into 20-mL XTB medium (20 g/L xylose, 24 g/L yeast extract, 12 g/L tryptone, 2.31 g/L KH_2PO_4, 16.43 g/L K_2HPO_4) in which xylose was

Fig. 1 Biosynthetic pathways of GABA from xylose in *E. coli* used in this study. The isomerase pathway of *E. coli* is shown in *black* and the introduced Weimberg pathway in *blue*. Chemical structures of metabolites from xylose to GABA via Weimberg pathway were shown. *Bold arrows* indicate the reactions enhanced by overexpressing the relevant genes. The reactions blocked by deleting the relevant genes is indicated by *cross*
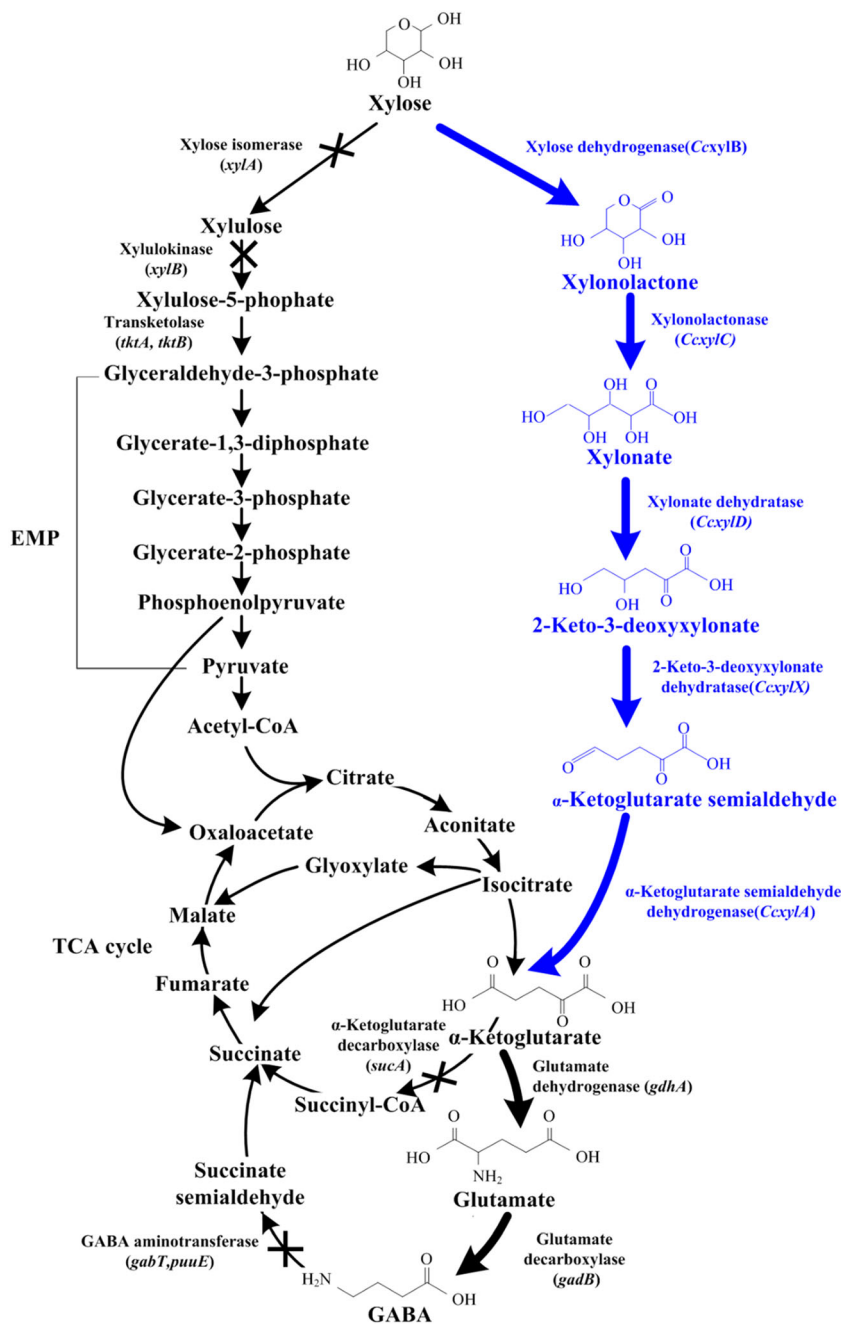
separately sterilized to avoid Maillard reaction, and incubated for 48 h at 37 °C and 200 rpm. The pH was kept to 6.5–7.5 by 10% (*v/v*) ammonia solution during the first 36 h, and 1 mM IPTG was added when OD_{600} reached 1.0. At 36 h, 10 mM PLP was added and pH was adjusted to 4.6 by 4 M HCl to produce GABA.

Construction of plasmids pWZtac-g2, pWZt7-g2, pWZt7-g3, pWZtac-xyl, and pWZt7-xyl

Restrictions enzymes, T4 DNA ligase and DNA ladder were purchased from Fermentus (USA). Prime STAR™ HS DNA polymerase was purchased from TaKaRa (Dalian, China). Kits for plasmids isolation, chromosomal DNA extraction, and DNA purification from agarose gels were purchased from Tiangen (China). DNA sequencings were performed by BGI (China). PCR experiments were performed by using Mastercycler from Eppendorf (Hamburg, Germany). The primers used in this study are listed in Table 2. Generally, 50-μL PCR reaction mixture includes 10 μL PS buffer, 4 μL dNTP mixture, 1 μL template (100 ng/μL), 1 μL forward primer (20 μM), 1 μL reverse primer (20 μM), and 0.5 μL PrimeSTAR™ HS DNA polymerase. PCR amplification was performed for 35 cycles. Each cycle consisted of

Table 1 Strains and main plasmids used in this study.

Names	Description	Source
Strains		
JM109	*E. coli* strain, F′ traD36 △(lacZ)M15/△(lac-proAB) lacIq proA⁺B⁺ glnV44 e14-gyrA96 recA1 end A1 thi hsdR17	Novagen
W3110	Wild type *E. coli*, ATCC27325	ATCC
WWZ03	W3110 derivation, deleting genes *puuE*, *gabT*, and *gabP*	This study
WWZ04	W3110 derivation, deleting genes *puuE*, *gabT*, *gabP*, and *sucA*	This study
WWZ06	W3110 derivation, deleting genes *puuE*, *gabT*, *gabP*, *sucA*, *xylA*, and *xylB*	This study
WWZ08	W3110 derivation, deleting genes *puuE*, *gabT*, *gabT*, *sucA*, *xylA* *xylB*, *waaF*, and *waaC*	This study
JWZ02	JM109 derivation, deleting genes *waaF* and *waaC*	This study
JWZ03	JM109 derivation, deleting genes *waaF*, *waaC*, and *sucA*	This study
JWZ06	JM109 derivation, deleting genes *waaF*, *waaC*, *sucA*, *puuE*, *gabT*, and *gabP*	This study
JWZ08	JM109 derivation, deleting genes *waaF*, *waaC*, *sucA*, *puuE*, *gabT*, *gabP*, *xylA*, and *xylB*	This study
BL21(DE3)	*E. coli* strain, F⁻ ompT gal dcm lon hsdS_B(r_B⁻ m_B⁻) λ (DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Novagen
ATCC13869	Wild type *C. glutamicum*	ATCC
NA1000	*C. crescentus* CB15N, derived from *C. crescentus* CB15 (ATCC 19089)	Evinger and Agabian (1977)
Plasmids		
pETDuet-1	Double T7 promoter, pBR322 ori, Ampʳ	Novagen
pETDuet-1-*CcxylB*	pETDuet-1 harboring *CcxylB* from *C. crescentus* NA1000 under T7 promoter	This study
pETDuet-1-*CcxylX*	pETDuet-1 harboring *CcxylX* from *C. crescentus* NA1000 under T7 promoter	This study
pETDuet-1-*CcxylD*	pETDuet-1 harboring *CcxylD* from *C. crescentus* NA1000 under T7 promoter	This study
pETDuet-1-*CcxylC*	pETDuet-1 harboring *CcxylC* from *C. crescentus* NA1000 under T7 promoter	This study
pETDuet-1-*CcxylA*	pETDuet-1 harboring *CcxylA* from *C. crescentus* NA1000 under T7 promoter	This study
pETDuet-1-*CcxylBC*	pETDuet-1 harboring *CcxylB* and *CcxylC* from *C. crescentus* NA1000 under T7 promoter	This study
pETDuet-1-*CcxylBCDC*	pETDuet-1 harboring *CcxylB*, *CcxylX*, *CcxylD*, and *CcxylC* from *C. crescentus* NA1000 under T7 promoter	This study
pACYCDuet-1	Double *T7* promoter, P15A *ori*, Cmʳ	Novagen
pACYCDuet-1-*gdhA*	pACYCDuet-1 harboring *gdhA* under t7 promoter	This study
pACYCDuet-1-*gadB*	pACYCDuet-1 harboring *gadB* under t7 promoter	This study
pDXW-8	*tac* promoter, pBR322 *ori*, Ampʳ and Kanʳ	Xu et al. (2010)
pDXW-8-*gdhA*	pDXW-8 harboring *gdhA* under *tac* promoter	This study
pDXW-8-*gadB*	pDXW-8 harboring *gadB* under *tac* promoter	This study
pDXW-8-*ccxylB*	pDXW-8 harboring *CcxylB* from *C. crescentus* NA1000 under *tac* promoter	This study
pDXW-8-*ccxylX*	pDXW-8 harboring *CcxylX* from *C. crescentus* NA1000 under *tac* promoter	This study
pDXW-8-*ccxylD*	pDXW-8 harboring *CcxylD* from *C. crescentus* NA1000 under *tac* promoter	This study
pDXW-8-*ccxylC*	pDXW-8 harboring *CcxylC* from *C. crescentus* NA1000 under *tac* promoter	This study
pDXW-8-*ccxylA*	pDXW-8 harboring *CcxylA* from *C. crescentus* NA1000 under *tac* promoter	This study
pDXW-8-*CcxylBX*	pDXW-8 harboring *CcxylB* and *CcxylX*, from *C. crescentus* NA1000 under *tac* promoter	This study
pDXW-8-*CcxylBXD*	pDXW-8 harboring *CcxylB*, *CcxylX*, and *CcxylD* from *C. crescentus* NA1000 under *tac* promoter	This study
pWZt7-xyl	pETDuet-1 harboring *CcxylB*, *CcxylX*, *CcxylD*, *CcxylC*, and *CcxylA* from *C. crescentus* NA1000 under T7 promoter	This study
pWZtac-xyl	pDXW-8 harboring *CcxylB*, *CcxylX*, *CcxylD*, *CcxylC*, and *CcxylA* from *C. crescentus* NA1000 under *tac* promoter	This study
pWZt7	pACYCDuet-1 deviation, P15A *ori*, Cmʳ, *T7* RNA polymerase under *ftsZ* promoter	This study
pWZt7-g2	pWZt7 harboring *gdhA* and *gadB* under T7 promoter	This study
pWZtac-g2	pACYCDuet-1 harboring *gdhA* and *gadB* under *tac* promoter	This study
pWZt7-g3	pWZt7 harboring *gdhA* and *torA-gadB* under T7 promoter	This study
pKD46	P_araBγβ exo, Repᵗˢ,Ampʳ	Datsenko and Wanner (2000)

Table 1 (continued)

Names	Description	Source
pKDCre	ParaB *cre*, *repA*(Ts) Amp^r	Han et al. (2013)
pDTW202	loxPLE-*kan*-loxPRE, Amp^r, Kan^r	Han et al. (2013)
pET20b-*torA*-*gadB*	pET20b(+) harboring *gadB* with signal peptide *torA*, Amp^r	Zhao et al. (2016)
pBlueScript II SK+	*E. coli* cloning vector, Amp^r	Stratagene
pBS-△*puuE*	pBlueScript II SK+ harboring *loxpLE-kan-loxpRE*, the upstream and downstream fragments of *puuE*	This study
pBS-△*gabTP*	pBlueScript II SK+ harboring *loxpLE-kan-loxpRE*, the upstream and downstream fragments of *gabTP*	This study
pBS-△*sucA*	pBlueScript II SK+ harboring *loxpLE-kan-loxpRE*, the upstream and downstream fragments of *puuE*	This study
pBS-△*xylAB*	pBlueScript II SK+ harboring *loxpLE-kan-loxpRE*, the upstream and downstream fragments of *sucA*	This study
pBS-△*waaFC*	pBlueScript II SK+ harboring *loxpLE-kan-loxpRE*, the upstream and downstream fragments of *waaFC*	This study

denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C. The time extension varied with the length of PCR product (1 kb/min).

The plasmid pACYCDuet-1 was used to construct pWZtac-g2, pWZt7-g2, and pWZt7-g3 (Fig. 2). pACYCDuet-1 contains two multiple cloning sites (MCS) preceded by T7 promoter.

To construct pWZtac-g2, the gene *gdhA* was PCR amplified by using primer pairs *gdhA*(tac)-F and *gdhA*(tac)-R and genomic DNA of *E. coli* W3110 as template, digested with *Eco*R I and *Hin*d III, and ligated into pDXW-8 (Xu et al. 2010) that was similarly digested; then, the fragment *ptac-gdhA* was PCR amplified by using primer pairs *Ptac-gdhA*-F and *Ptac-gdhA*-R from pDXW-8-*gdhA*, and digested with *Pst* I and *Afl* II. Similarly, the gene *gadB* was PCR amplified by using primer pairs *gadB*(tac)-F and *gadB* (tac)-R from the *E. coli* genomic DNA, digested with *Nhe* I and *Hin*d III, ligated into pDXW-8, and the fragment *ptac-gadB* was then PCR amplified by using primer pairs *Ptac-gadB*-F and *Ptac-gadB*-R, and digested with *Afl* II and *Kpn* I. The fragment *pACYCDuet-1-a* which contained no T7 promoters and MCSs was PCR amplified from the vector pACYCDuet-1, using the primer pairs *pACYC*-F and *pACYC*-R2, and digested with *Pst* I and *Kpn* I. The digested fragments *ptac-gdhA*, *ptac-gadB*, and *pACYCDuet-1-a* were ligated together to form the plasmid pWZtac-g2. pWZtac-g2 carried genes *gdhA* and *gadB* preceded by the *tac* promoters. Tac promoter is the combination of the *trp* and *lac* promoters but can be recognized by *E. coli* RNA polymerase more efficiently than either *trp* or *lac* promoters.

T7 promoter from *E. coli* bacteriophage T7 can be only recognized by T7 RNA polymerase. To express genes controlled by T7 promoters in *E. coli*, an *ftsZ* promoter-dependent T7 expression system was established (Equbal et al. 2013). This system expresses T7 RNA polymerase under the control

of *ftsZ* promoter and then T7 RNA polymerase transcribes the genes downstream the T7 promoter. A vector pWZt7 harboring gene encoding T7 RNA polymerase under the control of *ftsZ* promoter was constructed in this study. To construct pWZt7, the *ftsZ* promoter *pftsZ* was PCR amplified by using primer pairs *PftsZ*-F and *PftsZ*-R from the genomic DNA of *C. glutamicum* ATCC13869, and digested with *Pst* I and *Eco*R I; the gene *t7pol* encoding the T7 RNA polymerase was PCR amplified by using primer pairs *t7pol*-F and *t7pol*-R from the genomic DNA of *E. coli* BL21(DE3), and digested with *Eco*R I and *Bam*H I; the fragment *pACYCDuet-1-b* which contained no MCSs and T7 promoters was PCR amplified by using primer pairs *pACYC*-F and *pACYC*-R from the plasmid pACYCDuet-1, and digested with *Pst* I and *Bam*H I. Then, the digested fragment *pftsZ*, *t7pol*, and *pACYCDuet-1-b* were ligated together to form pWZt7. To construct pWZt7-g2, the gene *gdhA* was PCR amplified by using primer pairs *gdhA*(t7)-F and *gdhA*(t7)-R, digested with *Nco* I and *Sal* I, and ligated into pACYCDuet-1 that was similarly digested; then, the fragment *pt7-gdhA-teminater* was PCR amplified by using primers *Pt7-gdhA*-F and *Pt7-gdhA*-R, digested with *Pst* I and *Sca* I, and ligated into pWZt7 that was similarly digested, forming pWZt7-gdhA. The gene *gadB* was PCR amplified by using primer pairs *gadB*(t7)-F and *gadB*(t7)-R, digested with *Nde* I and *Kpn* I, and ligated into pACYCDuet-1 that was similarly digested; then, the fragment *pt7-gadB* was PCR amplified by using primer pairs *Pt7-gadB*-F and *Pt7-gadB*-R, digested with *Hin*d III and *Xho* I, and ligated into pWZt7-*gdhA* that was similarly digested, forming the plasmid pWZt7-g2. The fragment *pt7-torA-gadB* was PCR amplified by using primer pairs *Pt7-gadB*-F2 and *Pt7-gadB*-R2 from the plasmid pET20b-*torA*-*gadB* (Zhao et al. 2016), digested with *Hin*d III and *Xho* I, and ligated into pWZt7-*gdhA* that was similarly digested, forming the plasmid pWZt7-g3. The fragment *torA*

Table 2 Primers and their sequences used for PCR in this study

Names	5′–3′ sequences	Restriction site
Primers for the construction of pWZtac-g2		
gdhA(tac)-F	CGGAATTCAGAAGGAGATATACCATCAGACATATTCTCTGGAGTC	EcoR I
gdhA(tac)-R	CCCAAGCTTTTTAAATCACACCCTGCGCCA	Hind III
Ptac-gdhA-F	AAAACTGCAGTCGGAAGCTGTGGTATGG	Pst I
Ptac-gdhA-R	AATGCTTAAGCATCCGCCAAAACAGAAGCTT	Afl II
gadB(tac)-F	CTACGGCTAGCAGAAGGAGATATACCATGGATAAGAAGCAAGTAACG	Nhe I
gadB(tac)-R	CCCAAGCTTTAAGGTATGTTTAAAGCTGTT	Hind III
Ptac-gadB-F	AATGCTTAAGTCGGAAGCTGTGGTATGG	Afl II
Ptac-gadB-R	CGGGGTACCAGAGTTTGTAGAAACGCAAAAAGGCC	Kpn I
pACYC-R2	CGGGTACCCTAATGCAGGAGTCGCATAAGG	Kpn I
Primers for the construction of pWZt7-g2 and pWZt7-g3		
PftsZ-F	AATCTGCAGAGAAGTGCTTCCTGCGGT	Pst I
PftsZ-R	AATGAATTCCTCGCCTTTCGAAGAGTTG	EcoR I
t7pol-F	CGGAATTCATGAACACGATTAACATCGCTAAG	EcoR I
t7pol-R	CGGGATCCGGAGTCGTATTGATTTGGCG	BamH I
pACYC-F	AATCTGCAGACCGGTAAACCAGCAATAGACA	Pst I
pACYC-R	CGGGATCCCTAATGCAGGAGTCGCATAAGG	BamH I
gdhA(t7)-F	ATACCATGGATCAGACATATTCTCTGGAGT	Nco I
gdhA(t7)-R	ACGCGTCGACTTAAATCACACCCTGCGCCA	Sal I
Pt7-gdhA-F	AACAGTACTGCGATGAGTGGCAGGGC	Sca I
Pt7-gdhA-R	GCACTGCAGCGGGATCTCGACGCTCTCCC	Pst I
gadB(t7)-F	CGCCATATGGATAAGAAGCAAGTAACG	Nde I
gadB(t7)-R	ATAGGTACCTAAGGTATGTTTAAAGCTGTT	Kpn I
Pt7-gadB-F	CGACAAGCTTGCGGCCGCATAATGC	Hind III
Pt7-gadB-R	CAGACTCGAGGGTACCTAAGGTATGTTTAAAGCT	Xho I
Pt7-gadB-F2	CGACAAGCTTCGGGTCCTCAACGACAGGAG	Hind III
Pt7-gadB-R2	CAGACTCGAGTAAGGTATGTTTAAAGCTGTT	Xho I
Primers for the construction of pWZtac-xyl		
CcxylA(tac)-F	CGGAATTCAGAAGGAGATATACCATGACCGACACCCTGCGCCATT	EcoR I
CcxylA(tac)-R	AAAACTGCAGTTACGACCACGAGTAGGAGGTTTTGG	Hind III
Ptac-CcxylA-F	AATGCTGCAGTCGGAAGCTGTGGTATGG	Pst I
CcxylB(tac)-F	CGGAATTCAGAAGGAGATATACCATGGGCATGTCCTCAGCCATCTATCCCAG	EcoR I
CcxylB(tac)-R	CGAGCTCTCAACGCCAGCCGGCGTCGAT	Sac I
CcxylC(tac)-F	CGGAATTCAGAAGGAGATATACCATGACCGCTCAAGTCACTTGCG	EcoR I
CcxylC(tac)-R	AAAACTGCAGTTAGACAAGGCGGACCTCATGCT	Pst I
Ptac-CcxylC-F	AATGCTTAAGTCGGAAGCTGTGGTATGG	Afl II
CcxylD(tac)-F	CGGAATTCAGAAGGAGATATACCATGTCTAACCGCACGCCCC	EcoR I
CcxylD(tac)-R	AATGCTTAAGTTAGTGGTTGTGGCGGGGCAGC	Afl II
Ptac-CcxylD-F	CGGGGTACCTCGGAAGCTGTGGTATGG	kpn I
CcxylX(tac)-F	CTACGGCTAGCAGAAGGAGATATACCATGGGCGTGAGTGAATTCCTGC	Nhe I
CcxylX(tac)-R	ATACCATGGTTAGAGGAGGCCGCGGCCGG	Nco I
Ptac-CcxylX-F	CGAGCTCTCGGAAGCTGTGGTATGG	Sac I
Primers for the construction of pWZt7-xyl		
CcxylA(t7)-F	CGCCATATGATGACCGACACCCTGCGCCATT	Nde I
CcxylA(t7)-R	ATAGGTACCTTACGACCACGAGTAGGAGGTTTTGG	Kpn I
Pt7-CcxylA-F	ATAGGTACCTCGAACAGAAAGTAATCGTATTGTACAC	Kpn I
Pt7-CcxylA-R	CTGCCTAGGTTACGACCACGAGTAGGAGGTTTTGG	Avr II
CcxylB(t7)-F	CATGCCATGGGCATGTCCTCAGCCATCTATCCCAG	Nco I
CcxylB(t7)-R	CGCGGATCCTCAACGCCAGCCGGCGTCGAT	BamH I

Table 2 (continued)

| Names	5′–3′ sequences	Restriction site
CcxylC(t7)-F	CGCCATATGACCGCTCAAGTCACTTGCG	*Nde* I
CcxylC(t7)-R	ATAGGTACCTTAGACAAGGCGGACCTCATGCT	*Kpn* I
CcxylD(t7)-F	CGCCATATGTCTAACCGCACGCCCC	*Nde* I
CcxylD(t7)-R	ATAGGTACCTTAGTGGTTGTGGCGGGGCAGC	*Kpn* I
Pt7-CcxylD-F	AATGCTTAAGTCGAACAGAAAGTAATCGTATTGTACAC	*Afl* II
Pt7-CcxylD-R	CCCAAGCTTTTAGTGGTTGTGGCGGGGCAGC	*Hin*d III
CcxylX(t7)-F	CATGCCATGGGCGTGAGTGAATTCCTGC	*Nco* I
CcxylX(t7)-R	CGCGGATCCTTAGAGGAGGCCGCGGCCGG	*Bam*H I
Pt7-CcxylX-F	GCACTGCAGCGGGATCTCGACGCTCTCC	*Pst* I
Pt7-CcxylX-R	AATGCTTAAGTTAATTAGAGGAGGCCGCGGCCGG	*Afl* II
Primers used for gene deletions		
kan-F	ATGGATCCAATACGACTCACTATAGG	*Bam*H I
kan-R1	ACCTCTAGAGCGCAATTAACCCTCACTAAAG	*Xba* I
kan-R2	CCCAAGCTTCGCAATTAACCCTCACTAAAG	*Hin*d III
puuE-U-F	ACTCTCGAGCATCCGCTAAACCTTGCCA	*Xho* I
puuE-U-R	ACCTCTAGACGGAAATTAAACGCCCAGA	*Xba* I
puuE-D-F	ACGGATCCAATTCGATGCGGCAATGAA	*Bam*H I
puuE-D-R	AAACTGCAGCGGCGGTACGCTATTTCTTG	*Pst* I
puuE-F	CATCCGCTAAACCTTGCCA	
puuE-R	ATTGCAGGTGAATGTGCGG	
gabTP-U-F	ACTCTCGAGCCCGGCGGCGATGATTA	*Xho* I
gabTP-U-R	ACCTCTAGATAAAGACCGATGCACATATATTTG	*Xba* I
gabTP-D-F	ACGGATCCGCTGGTATTGTGGCAAAAAAC	*Bam*H I
gabTP-D-R	AAACTGCAGTCAGGAGGCGAAGGAGAAA	*Pst* I
gabTP-F	CCCGGCGGCGATGATTA	
gabTP-R	TCAGGAGGCGAAGGAGAAA	
sucA-U-F	CGGGATCCAATTTATCGCTATAACCCGGATG	*Bam*H I
sucA-U-R	CGGAATTCTTCTATCCAGCTCTGGTTTGC	*Eco*R I
sucA-D-F	CCCAAGCTTCAAGATCTGGTTAATGACGCG	*Hin*d III
sucA-D-R	GCACTGCAGAACACACCACCGTTGGTGAT	*Pst* I
sucA-F	AATTTATCGCTATAACCCGGATG	
sucA-R	AACACACCACCGTTGGTGAT	
xylAB-U-F	CGGGATCCTTATCTACCGGCGAGCCGCC	*Bam*H I
xylAB-U-R	ACCAAGCTTACGAACGCGATCGAGCTGGTC	*Hin*d III
xylAB-D-F	CGGAATTCACGTTATCCCCTGCCTGACCG	*Eco*R I
xylAB-D-R	CTACTGCAGGCCTTCATACTTGTCCCGAACGG	*Pst* I
xylAB-F	TTATCTACCGGCGAGCCGCC	
xylAB-R	GCCTTCATACTTGTCCCGAACGG	
waaFC-U-F	AAAACTGCAGTGCTACGCTGGCTTATC	*Pst* I
waaFC-U-R	CCCAAGCTTAGCTCTTATGCGTCGCGATTCAG	*Hin*d III
waaFC-D-F	CGGAATTCCAGTCAAGCAGTTTTGGA	*Eco*R I
waaFC-D-R	CCGCTCGAGTAAATCAAGCAAGCCTAT	*Xho* I
waaFC-F	TGCTACGCTGGCTTATC	
waaFC-R	TAAATCAAGCAAGCCTAT	

The restriction sites are underlined

encodes a signal peptide which can facilitate the secretion of GadB encoded by *gadB* (Fig. 2).

Both pDXW-8 (Xu et al. 2010) and pETDuet-1 harbor pBR322 replicon. pDXW-8 carries one MCS under the

control of tac promoter. pETDuet-1 carries two MCSs, each of which was preceded by a T7 promoter. pDXW-8 and pETDuet-1 were used to construct pWZtac-xyl and pWZt7-xyl, respectively. To construct pWZtac-xyl, the genes *CcxylB*, *CcxylX*, *CcxylD*, *CcxylC*, and *CcxylA* were PCR amplified from the genomic DNA of *C. crescentus* NA1000, respectively, by using primer pairs *CcxylB*(tac)-F and *CcxylB*(tac)-R, *CcxylX*(tac)-F and *CcxylX*(tac)-R, *CcxylD*(tac)-F and *CcxylD*(tac)-R, *CcxylC*(tac)-F and *CcxylC*(tac)-R, and *CcxylA*(tac)-F and *CcxylA*(tac)-R. The PCR products of genes

CcxylB, *CcxylX*, *CcxylD*, *CcxylC*, and *CcxylA* were digested with *Eco*R I and *Sac* I, *Nhe* I and *Nco* I, *Eco*R I and *Afl* II, *Eco*R I and *Pst* I, and *Eco*R I and *Hin*d III, respectively, and ligated into pDXW-8 that was similarly digested, forming the plasmids pDXW-8-*CcxylB*, pDXW-8-*CcxylX*, pDXW-8-*CcxylD*, pDXW-8-*CcxylC*, and pDXW-8-*CcxylA*. The fragment *ptac-CcxylX* was PCR amplified by using *Ptac-CcxylX*-F and *CcxylX*(tac)-R from pDXW-8-*CcxylX*, digested with *Sac* I and *Nco* I, and ligated into pDXW-8-*CcxylB* similarly digested, forming the plasmid pDXW-8-*CcxylBX*. The

Fig. 2 Maps of plasmids pACYCDuet-1, pETDuet-1, pDXW-8, pWZt7-xyl, pWZtac-xyl, pWZt7-g2, pWZt7-g3, and pWZtac-g2
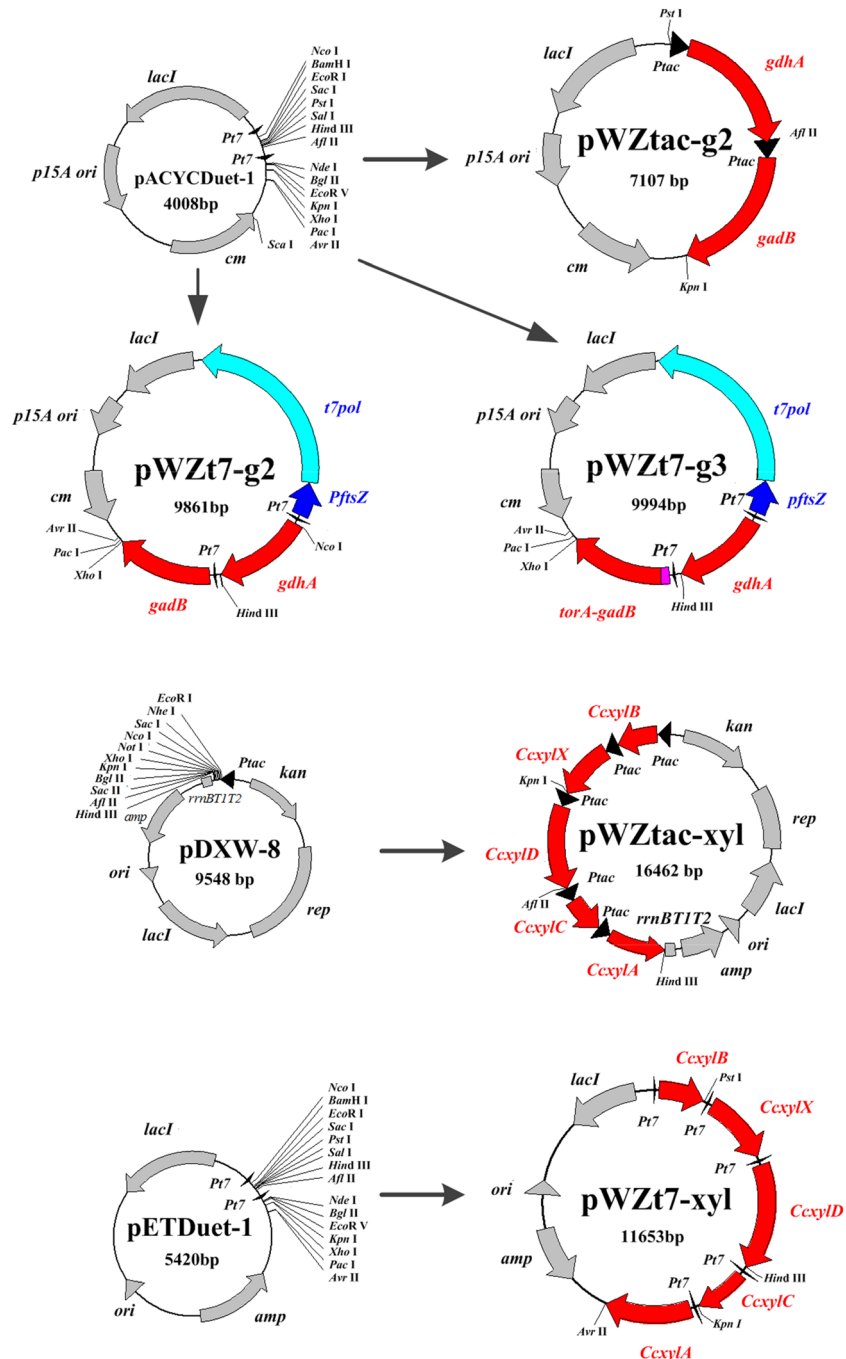
fragment *ptac-CcxylD* was PCR amplified by using primer pairs *Ptac-CcxylD*-F and *CcxylD*(tac)-R from pDXW-8-*CcxylD*, digested with *kpn* I and *Afl* II, and ligated into pDXW-8-*CcxylBX,* forming the plasmid pDXW-8-*CcxylBXD*. The fragment *ptac-CcxylC* amplified by using *Ptac-CcxylC*-F and *CcxylC*(tac)-R from pDXW-8-*CcxylC* was digested with *Afl* II and *Pst* I; the fragment *ptac-CcxylA* amplified by using *Ptac-CcxylA*-F and *CcxylA*(tac)-R from pDXW-8-*CcxylA* was digested with *Pst* I and *Hin*d III. The digested fragment *ptac-CcxylC* and *ptac-CcxylA* were ligated into pDXW-8-*CcxylBXD* digested with *Afl* II and *Hin*d III, forming the plasmid pWZtac-xyl. pWZtac-xyl could co-exist with pWZtac-g2 in the same *E. coli* cell.

To construct pWZt7-xyl, the genes *CcxylB*, *CcxylX*, *CcxylD*, *CcxylC*, and *CcxylA* were PCR amplified from the genomic DNA of *C. crescentus* NA1000, respectively, by using primer pairs *CcxylB*(t7)-F and *CcxylB*(t7)-R, *CcxylX*(t7)-F and *CcxylX*(t7)-R, *CcxylD*(t7)-F and *CcxylD*(t7)-R, *CcxylC*(t7)-F and *CcxylC*(t7)-R, and *CcxylA*(t7)-F and *CcxylA*(t7)-R. The PCR products of genes *CcxylB*, *CcxylX*, *CcxylD*, and *CcxylA* were digested with *Nco* I and *Bam*H I, *Nco* I and *Bam*H I, *Nde* I and *Kpn* I, and *Nde* I and *Kpn* I, respectively, and ligated into pETDuet-1 that was similarly digested, forming the plasmids pETDuet-1-*CcxylB*, pETDuet-1-*CcxylX*, pETDuet-1-*CcxylD*, and pETDuet-1-*CcxylA*. The PCR product of the gene *CcxylC* was digested with *Nde* I and *Kpn* I, and ligated into the pETDuet-1-*CcxylB* that was similarly digested, forming the plasmid pETDuet-1-*CcxylBC*. The fragment *pt7-CcxylX* was PCR amplified by using *Pt7-CcxylX*-F and *Pt7-CcxylX*-R from plasmid pETDuet-1-*CcxylX*, and digested with *Pst* I and *Afl* II; the fragment *pt7-CcxylD* was PCR amplified by using *Pt7-CcxylD*-F and *Pt7-CcxylD*-R form plasmid pETDuet-1-*CcxylD*, and digested with *Afl* II and *Hin*d III. The digested fragments *pt7-CcxylX* and *pt7-CcxylD* were ligated into pETDuet-1-*CcxylBC* which digested with *Pst* I and *Hin*d III, forming the plasmid pETDuet-1-*CcxylBXDC*. The fragment *pt7-CcxylA* was PCR amplified by using *Pt7-CcxylA*-F and *Pt7-CcxylA*-R from pETDuet-1-*CcxylA*, digested with *kpn* I and *Avr* II, and ligated into pETDuet-1-*CcxylBXDC* which was similarly digested, forming the plasmid pWZt7-xyl.

Multiple gene deletions in *E. coli* JM109 and W3110

Multiple genes were deleted in *E. coli* JM109 and W3110, using Red recombination system (Datsenko and Wanner 2000), and the resistance genes were removed by using Cre-loxP system (Han et al. 2013).

In *E. coli* W3110, the gene *puuE* was firstly deleted. The upstream fragment of *puuE* was PCR amplified by using primer pairs *puuE*-U-F and *puuE*-U-R from the genomic DNA of *E. coli* W3110, and digested with *Xho* I and *Xba* I; the downstream fragment of *puuE* was PCR amplified by using primer

pairs *puuE*-D-F and *puuE*-D-R, and digested by *Bam*H I and *Pst* I; the fragment *loxpLE-kan-loxpRE* was PCR amplified by using primer pairs *kan*-F and *kan*-R1 from the plasmid pDTW202 (Han et al. 2013), and digested by *Xba* I and *Bam*H I. The three digested PCR products were ligated into pBluescript II SK(+)digested with *Xho* I and *Pst* I, and then the DNA segment containing the *loxpLE-kan-loxpRE*, and the upstream and downstream fragments of *puuE* was PCR amplified by using primer pairs *puuE*-F and *puuE*-R, and transformed into *E. coli* W3110 cells harboring the Red recombination plasmid pKD46 to replace the gene *puuE* with *loxpLE-kan-loxpRE*. Then, the *kan* gene was removed by introducing the plasmid pKDCre which can overexpress recombinase Cre and recombine the *loxp* sequences at both ends of the *kan*, resulting in the mutant strain W3110△*puuE*. Both pKDCre and pKD46 are temperature sensitive; therefore, they can be cured by growing the cells at 42 °C. Similarly, genes *gabT* and *gabP* were deleted in W3110△*puuE*, resulting in WWZ03. Genes *gabT* and *gabP* are next to each other, so they were deleted by one recombination. The upstream fragment of *gabTP* was amplified by using primer pairs *gabTP*-U-F and *gabTP*-U-R, and the downstream fragment of *gabTP* was amplified by using primer pairs *gabTP*-D-F and *gabTP*-D-R. The DNA segment containing the *loxpLE-kan-loxpRE*, and the upstream and downstream fragments of *gabTP* was amplified by using primer pairs *gabTP*-F and *gabTP*-R. The gene *sucA* was deleted in WWZ03, resulting in WWZ04. The upstream fragment of *sucA* was amplified by using primer pairs *sucA*-U-F and *sucA*-U-R, the downstream fragment of *sucA* was amplified by using primer pairs *sucA*-D-F and *sucA*-D-R, and the fragment *loxpLE-kan-loxpRE* was amplified by using primer pairs *kan*-F and *kan*-R2. The DNA segment containing the *loxpLE-kan-loxpRE*, and the upstream and downstream fragments of *sucA* was amplified by using primer pairs *sucA*-F and *sucA*-R. The genes *xylA* and *xylB* were deleted in WWZ04, resulting in WWZ006. Genes *xylA* and *xylB* are next to each other on the chromosome, so they were deleted by one recombination. The upstream fragment of *xylAB* was amplified by using primer pairs *xylAB*-U-F and *xylAB*-U-R, the downstream fragment of *xylAB* was amplified by using primer pairs *xylAB*-D-F and *xylAB*-D-R. The DNA segment containing the *loxpLE-kan-loxpRE*, and the upstream and downstream fragments of *xylAB* was amplified by using primer pairs *xylAB*-F and *xylAB*-R. The genes *waaC* and *waaF* were deleted in WWZ06, resulting in WWZ008. Genes *waaC* and *waaF* were contiguous, and they were deleted by one recombination. The upstream fragment of *waaFC* was amplified by using primer pairs *waaFC*-U-F and *xylAB*-U-R, and the downstream fragment of *waaFC* was amplified by using primer pairs *waaFC*-D-F and *waaFC*-D-R. The DNA segment containing the *loxpLE-kan-loxpRE*, and the upstream and downstream fragments of *waaFC* was amplified by using primer pairs *waaFC*-F and *waaFC*-R.

Using the similar protocol, the eight genes *puuE*, *gabT*, *gabP*, *sucA*, *xylA*, *xylB*, *waaF*, and *waaC* were also deleted in *E. coli* JM109. Genes *waaF* and *waaC* were first deleted in JM109, resulting in the mutant JWZ02; the gene *sucA* in JWZ02 was deleted, resulting in the mutant JWZ03; genes *puuE*, *gabT*, and *gabP* in JWZ03 were deleted, resulting the mutant JWZ06; finally, genes *xylA* and *xylB* in JWZ06 were deleted, resulting in the mutant JWZ08.

Construction of *E. coli* recombinant strains

Recombinant strains JM109/pWZt7-g2 and W3110/pWZt7-g2 were constructed by introducing pWZt7-g2 into JM109 and W3110, respectively. JM109/pWZtac-g2 and W3110/pWZtac-g2 were constructed by introducing pWZtac-g2 into JM109 and W3110, respectively. By introducing both pWZt7-g2 and pWZt7-xyl into JM109, JWZ02, JWZ03, JWZ06, JWZ08, W3110, WWZ03, WWZ04, WWZ06, and WWZ08, respectively, recombinant strains JM109/pWZt7-g2/pWZt7-xyl, JWZ02/pWZt7-g2/pWZt7-xyl, JWZ03/pWZt7-g2/pWZt7-xyl, JWZ06/pWZt7-g2/pWZt7-xyl, JWZ08/pWZt7-g2/pWZt7-xyl, W3110/pWZt7-g2/pWZt7-xyl, WWZ03/pWZt7-g2/pWZt7-xyl, WWZ04/pWZt7-g2/pWZt7-xyl, WWZ06/pWZt7-g2/pWZt7-xyl, and WWZ08/pWZt7-g2/pWZt7-xyl were constructed. By introducing both pWZtac-g2 and pWZtac-xyl into JM109, JWZ02, JWZ03, JWZ06, JWZ08, W3110, WWZ03, WWZ04, WWZ06, and WWZ08, respectively, recombinant strains JM109/pWZtac-g2/pWZtac-xyl, JWZ02/pWZtac-g2/pWZtac-xyl, JWZ03/pWZtac-g2/pWZtac-xyl, JWZ06/pWZtac-g2/pWZtac-xyl, JWZ08/pWZtac-g2/pWZtac-xyl W3110/pWZtac-g2/pWZtac-xyl, WWZ03/pWZtac-g2/pWZtac-xyl, WWZ04/pWZtac-g2/pWZtac-xyl, WWZ06/pWZtac-g2/pWZtac-xyl, and WWZ08/pWZtac-g2/pWZtac-xyl were constructed. By introducing both pWZt7-g3 and pWZt7-xyl into JWZ02, JWZ03, JWZ06, and JWZ08, respectively, recombinant strains JWZ02/pWZt7-g3/pWZt7-xyl, JWZ03/pWZt7-g3/pWZt7-xyl, JWZ06/pWZt7-g3/pWZt7-xyl, and JWZ08/pWZt7-g3/pWZt7-xyl were constructed.

Analysis of cell density and levels of Glu, GABA, and xylose

Cell density of different *E. coli* strains was determined by measuring OD_{600} with UV-1800 spectrophotometer (Shimadzu, Japan).

Glu and GABA production in different cells were analyzed by HPLC (Agilent Technologies 1200 series, USA). *E. coli* cells were grown and centrifuged, the supernatant of broth was treated with 20 volume 5% (*w/v*) trichloroacetic acid for 2 h to precipitate proteins, and the clear filtrate was used directly for HPLC analysis. HPLC separation and quantitation was performed on a Thermo ODS C18 column (5 μm,

250 mm × 4.6 mm, USA) by using the *o*-phthalaldehyde (OPA) pre-column derivatization method (Kőrös et al. 2008). Separation of the samples was attained using a binary nonlinear gradient with solution A (sodium acetate 3.01 g, tetrahydrofuran 3 mL, and trimethylamine 0.2 mL dissolved or diluted in ddH_2O to 1 L, pH 7.2) and solution B (sodium acetate 3.01 g dissolved in ddH_2O to 200 mL, and then supplied with 400 mL methanol and 400 mL acetonitrile, pH 7.2). The column was kept at 40 °C. Elution conditions were as follows: equilibration (5 min, 8% B), gradient (15 min, 8–100% B), and cleaning (5 min, 100% B). The mobile phase was set at 1 mL/min. Glu and GABA were detected at 338 nm, and the spectra were recorded online.

The xylose concentration was determined by using the published method (Eberts et al. 1979) with modification. Briefly, 0.5 mL properly diluted supernatant of the broth was mixed with 2.5 mL phloroglucinol reagent (0.5 g phloroglucinol, 100 mL acetic acid, and 6 mL HCl), heated for 8 min at 100 °C, cooled in water to room temperature, and its absorbance at 554 nm was measured. Xylose concentration was calculated according to a standard curve.

Results

Weimberg pathway could be used in *E. coli* for GABA production from xylose

Two-stage pH control fermentation strategy was used in this study for GABA production from xylose in *E. coli*. During the first 36 h, pH was kept at 6.5 to 7.5, Glu was accumulated; after 36 h, pH was kept at 4.6, and GABA was produced; *E. coli* cells was viable in the first 36 h, but most cells could not survive during 36–48 h after pH was decreased. Therefore, Glu concentrations and cell densities (OD_{600}) at 36 h as well as GABA productions at 48 h were collected to value the performance of different *E. coli* mutant cells.

When xylose was used as the carbon source, wild-type *E. coli* W3110 grew well (OD_{600} at 36 h reached 19.71), but accumulated neither Glu at 36 h nor GABA at 48 h (Fig. 3). Bacteria including *E. coli* exist the gene *gdhA* encoding glutamate dehydrogenase Gdh and the gene *gadB* encoding glutamate decarboxylase GadB; Gdh converts α-ketoglutarate to Glu, and GadB converts Glu to GABA. Overexpressing Gdh in *C. glutamicum* could enhance Glu production (Asakura et al. 2007). Therefore, genes *gdhA* and *gadB* were inserted into the vector pACYCDuet-1, forming pWZt7-g2 (both genes were controlled by T7 promoters) and pWZtac-g2 (both genes were controlled by *tac* promoters), and transformed into *E. coli* W3110. 1.14 and 1.27 g/L Glu were accumulated after 36 h in the fermentation of W3110/pWZt7-g2 and W3110/pWZtac-g2 cells, respectively; 0.35 and 0.52 g/L GABA were produced after 48 h in the fermentation of W3110/pWZt7-g2

and W3110/pWZtac-g2 cells, respectively. Compared with the wild type, OD_{600} of W3110/pWZt7-g2 and W3110/pWZtac-g2 cells at 36 h slightly decreased, probably due to the metabolic burden of gene overexpression and plasmid replication (Wu et al. 2013).

$E.\ coli$ has an inherent pathway metabolizing xylose to α-ketoglutarate, but it is a long and complex pathway involving EMP and TCA cycle (Fig. 1). Weimberg pathway found in $C.\ crescentus$ NA1000 can covert xylose to α-ketoglutarate in five reactions. Weimberg pathway was implemented in $C.\ glutamicum$ for cell growth on xylose as sole carbon source (Radek et al. 2014), and also used to produce xylonate in $E.\ coli$ BL21(DE3) and W3110(DE3) by co-expressing $CcxylB$ and $CcxylC$ under the control of T7 promoter (Cao et al. 2013; Liu et al. 2012a). Therefore, the five Weimberg pathway genes $CcxylB$, $CcxylX$, $CcxylD$, $CcxylC$, and $CcxylA$ were either cloned into pDXW-8 under the control of tac promoter (pWZtac-xyl) or into pETDuet-1 under the control of tac promoter (pWZt7-xyl), and transformed into $E.\ coli$ W3110/WZt7-g2 and W3110/pWZtac-g2, respectively. Due to their different replicons, plasmids pWZt7-xyl and pWZt7-g2 could co-exist in the same $E.\ coli$ cells, so could the plasmids pWZtac-xyl and pWZtac-g2. Therefore, the recombinant $E.\ coli$ W3110/pWZt7-g2/pWZt7-xyl and W3110/pWZtac-g2/pWZtac-xyl cells could overexpress all seven enzymes required for GABA production from xylose. As expected, Glu concentrations at 36 h and GABA concentrations at 48 h further increased in W3110/pWZt7-g2/pWZt7-xyl and W3110/pWZtac-g2/pWZtac-xyl (Fig. 3), suggesting that Weimberg pathway could be used in $E.\ coli$ for GABA production from xylose.

The same plasmids pWZt7-g2, pWZt7-xyl, pWZtac-g2, and pWZtac-xyl were also transformed into $E.\ coli$ JM109. Neither Glu nor GABA was detected during two-stage pH control fermentation of $E.\ coli$ JM109. However, JM109/pWZt7-g2 and JM109/pWZtac-g2 produced about 1.2 g/L Glu at 36 h and 0.5 g/L GABA at 48 h; JM109/pWZt7-g2/pWZt7-xyl and JM109/pWZtac-g2/pWZtac-xyl produced about 1.9 g/L Glu at 36 h and 0.75 g/L GABA at 48 h (Fig. 3). The results demonstrate again that Weimberg pathway could be used in $E.\ coli$ for GABA production from xylose.

Multiple gene deletions in $E.\ coli$ W3110 to enhance GABA production

Gene $gabP$ encodes GABA transporter which transports extracellular GABA into $E.\ coli$ cells. Both genes $gabT$ and $puuE$ encode GABA aminotransferases which convert GABA to succinic semialdehyde. Succinic semialdehyde can be converted to succinate by succinic semialdehyde dehydrogenase. These enzymes play important role in the GABA shunt. Deletion of $gabP$, $gabT$, and $puuE$ should decrease the consumption of GABA by the cells. GABA production was enhanced in $E.\ coli$ when $gabT$ was deleted, using glucose as the carbon source (Pham et al. 2016a). In this study, the three genes $gabP$, $gabT$, and $puuE$ were deleted in $E.\ coli$ W3110, and plasmid pairs pWZt7-g2/pWZt7-xyl and pWZtac-g2/pWZtac-xyl were transformed into the triple deletion mutant WWZ03 (Fig. 4). After two-stage pH control fermentation, 1.04 and 0.93 g/L GABA were produced in WWZ03/pWZt7-g2/pWZt7-xyl and WWZ03/pWZtac-g2/pWZtac-xyl, respectively.

Gene $sucA$ encodes the subunit E1 of α-ketoglutarate decarboxylase which converts α-ketoglutarate to succinyl-CoA (Fig. 1). More Glu could be accumulated when $sucA$ was deleted in $E.\ coli$ or $C.\ glutamicum$ (Asakura et al. 2007; Hirasawa et al. 2012; Nishio et al. 2013). In this study, the gene $sucA$ was deleted in WWZ03, and the plasmid pairs pWZt7-g2/pWZt7-xyl and pWZtac-g2/pWZtac-xyl were
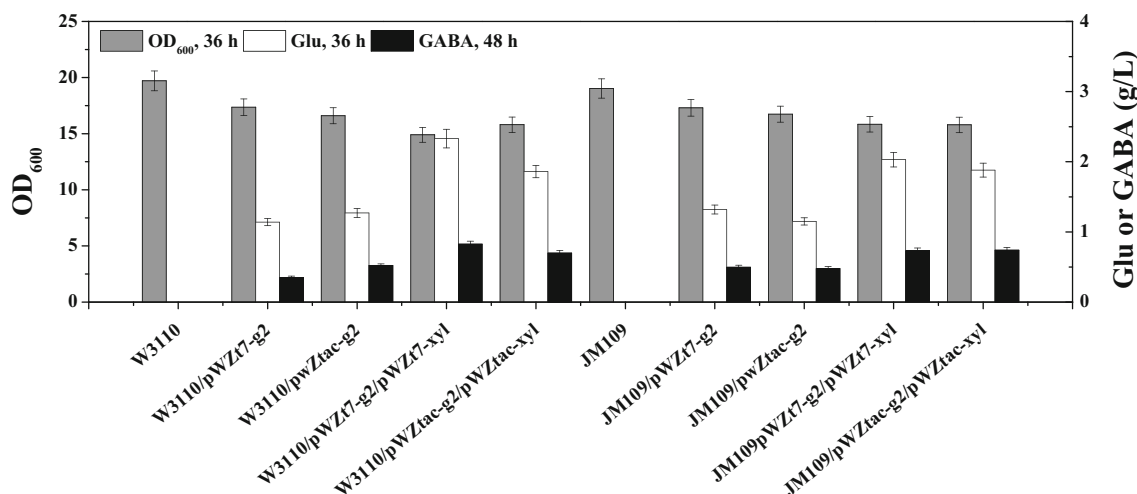

Fig. 3 Glu production at 36 h, OD_{600} at 36 h, and GABA production at 48 h after two-stage pH control fermentation in $E.\ coli$ strains W3110, JM109, W3110/pWZt7-g2, W3110/pWZtac-g2, JM109/pWZt7-g2, JM109/pWZtac-g2, W3110/pWZt7-g2/pWZt7-xyl, W3110/pWZtac-g2/pWZtac-xyl, JM109/pWZt7-g2/pWZt7-xyl, and JM109/pWZtac-g2/pWZtac-xyl

transformed into the quadruple deletion mutant WWZ04 (Fig. 4). After two-stage pH control fermentation, productions of both Glu (3.52 and 2.94 g/L) and GABA (1.97 and 1.66 g/L) significantly increased in WWZ04/pWZt7-g2/pWZt7-xyl and WWZ04/pWZtac-g2/pWZtac-xyl (Fig. 4). More than 80% Glu accumulated at 36 h were converted to GABA at 48 h. Compared with the control strain WWZ04, OD$_{600}$ of WWZ04/pWZt7-g2/pWZt7-xyl and WWZ04/pWZtac-g2/pWZtac-xyl at 36 h decreased. The cell growth inhibition might be due to the deletion of *sucA* which caused the breakdown of TCA cycle. The supply of oxaloacetate and succinate might rely on the glyoxylate cycle (Asakura et al. 2007).

In *E. coli*, genes *xylA* and *xylB* encoded xylose isomerase and xylulose kinase, respectively. Deletion of *xylA* and *xylB* and overexpression the first two genes of Weimberg pathway in *E. coli* increased xylonate production from xylose (Cao et al. 2013; Liu et al. 2012a). In this study, genes *xylA* and *xylB* were deleted in WWZ04 and the plasmid pairs pWZt7-g2/pWZt7-xyl and pWZtac-g2/pWZtac-xyl were transformed into the sextuple deletion mutant WWZ06 (Fig. 4). Unfortunately, productions of neither Glu nor GABA in WWZ06/pWZt7-g2/pWZt7-xyl and WWZ06/pWZtac-g2/pWZtac-xyl after two-stage pH

control fermentation were exceeded those in WWZ04/pWZt7-g2/pWZt7-xyl and WWZ04/pWZtac-g2/pWZtac-xyl (Fig. 4).

In *E. coli*, genes *waaC* and *waaF* encode heptosyltransferase WaaC and WaaF which sequentially add two L-D-heptoses to Kdo$_2$-lipid A of lipopolysaccharide in the outer membrane (Brabetz et al. 1997). Deletion of *waaC* and *waaF* could increase membrane permeability of *E. coli* (Wang et al. 2015). In this study, genes *waaC* and *waaF* were deleted in WWZ06 and the plasmid pairs pWZt7-g2/pWZt7-xyl and pWZtac-g2/pWZtac-xyl were transformed into the octuplet deletion mutant WWZ08 (Fig. 4). Production levels of Glu and GABA in WWZ08/pWZt7-g2/pWZt7-xyl and WWZ08/pWZtac-g2/pWZtac-xyl after two-stage pH control fermentation were similar to those in WWZ06/pWZt7-g2/pWZt7-xyl and WWZ06/pWZtac-g2/pWZtac-xyl (Fig. 4).

Multiple gene deletions in *E. coli* JM109 to enhance GABA production

Genes *waaC*, *waaF*, *gabP*, *gabT*, *puuE*, *sucA*, *xylA*, and *xylB* in *E. coli* JM109 were also deleted in different ways

Fig. 4 **a** Comparison of different deletion mutants derived from *E. coli* W3110. **b** Glu production at 36 h, OD$_{600}$ at 36 h, and GABA production at 48 h after two-stage pH control fermentation in *E. coli* strains WWZ3, WWZ3/WZt7-g2/pWZt7-xyl, WWZ3/pWZtac-g2/pWZt7-xyl, WWZ4, WWZ4/pWZt7-g2/pWZt7-xyl, WWZ4/pWZtac-g2/pWZt7-xyl, WWZ6, WWZ2/pWZt7-g6/pWZt7-xyl, WWZ6/pWZtac-g2/pWZt7-xyl, WWZ8, WWZ8/pWZt7-g2/pWZt7-xyl, and WWZ8/pWZtac-g2/pWZt7-xyl

as shown in Fig. 5a, resulting in deletion mutants JWZ02, JWZ03, JWZ06, and JWZ08. The plasmid pairs pWZt7-g2/pWZt7-xyl, pWZtac-g2/pWZtac-xyl, and pWZt7-g2/pWZt7-xyl were transformed into these deletion mutant cells, forming a series of recombinant *E. coli* strains. Plasmid pWZt7-g3 is similar to pWZt7-g2; the difference between them is that there is *torA* fused with *gadB* in pWZt7-g3 (Fig. 2). Signal peptide TorA encoded by *torA* could efficiently secrete GadB in *E. coli* to improve the production of GABA (Zhao et al. 2016).

Productions of Glu and GABA in different recombinant *E. coli* strains after two-stage pH control fermentation are shown in Fig. 5b. Generally, *E. coli* cells harboring plasmid pairs containing T7 promoters produced more Glu and GABA than those cells harboring plasmid pairs containing *tac* promoters. JWZ08 cells produced more Glu and GABA than other cells when harboring the same plasmid pairs, and the most GABA production was obtained in the strain JWZ08/pWZt7-g3/pWZt7-xyl. After two-stage pH control fermentation of JWZ08/pWZt7-g3/pWZt7-xyl, 6.25 g/L Glu at 36 h, and 3.21 g/L GABA was produced at 48 h.

Optimize GABA production in JWZ08/pWZt7-g3/pWZt7-xyl

Among all the recombinant *E. coli* strains constructed in this study, the maximum GABA production was obtained in the strain JWZ08/pWZt7-g3/pWZt7-xyl. Therefore, the optimum conditions for the fermentation of JWZ08/pWZt7-g3/pWZt7-xyl to produce GABA were performed.

The optimum temperature for wild *E. coli* growth is 37 °C, but some engineered *E. coli* grew better at 30 °C. In this study, at 30 °C, OD_{600} at 36 h of JWZ08/pWZt7-g3/pWZt7-xyl was 8.30; Glu concentration at 36 h and GABA production at 48 h were 4.50 and 1.26 g/L, respectively. Comparing the performance of JWZ08/pWZt7-g3/pWZt7-xyl at 37 °C, the cell growth was similar, but Glu and GABA productions at 30 °C were 28 and 61% lower than those at 37 °C (Fig. 6a).

In *E. coli* BL21(DE3), IPTG is required to express T7 RNA polymerase which is under control of the IPTG-inducible *lacUV5* promoter. In this study, the *ftsZ* promoter-dependent T7 expression system was used, which is not induced by IPTG (Equbal et al. 2013). However, in pWZt7-g2, pWZt7-g3 and
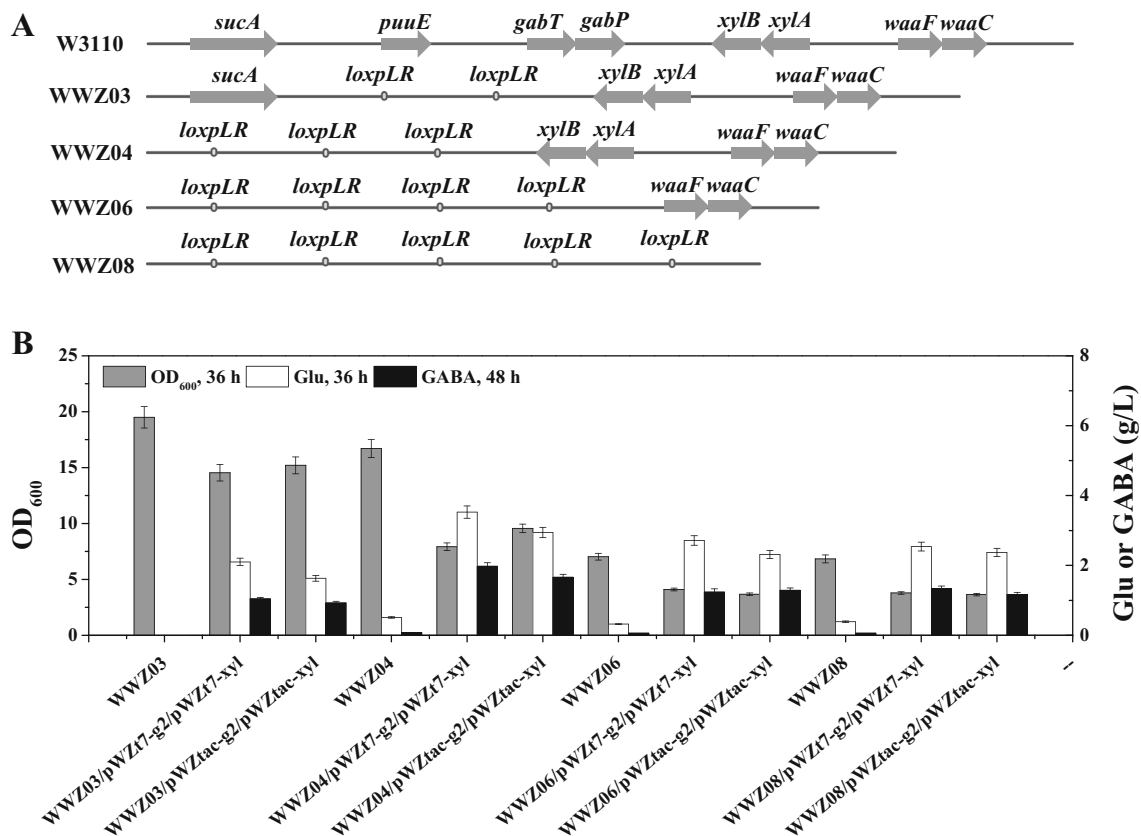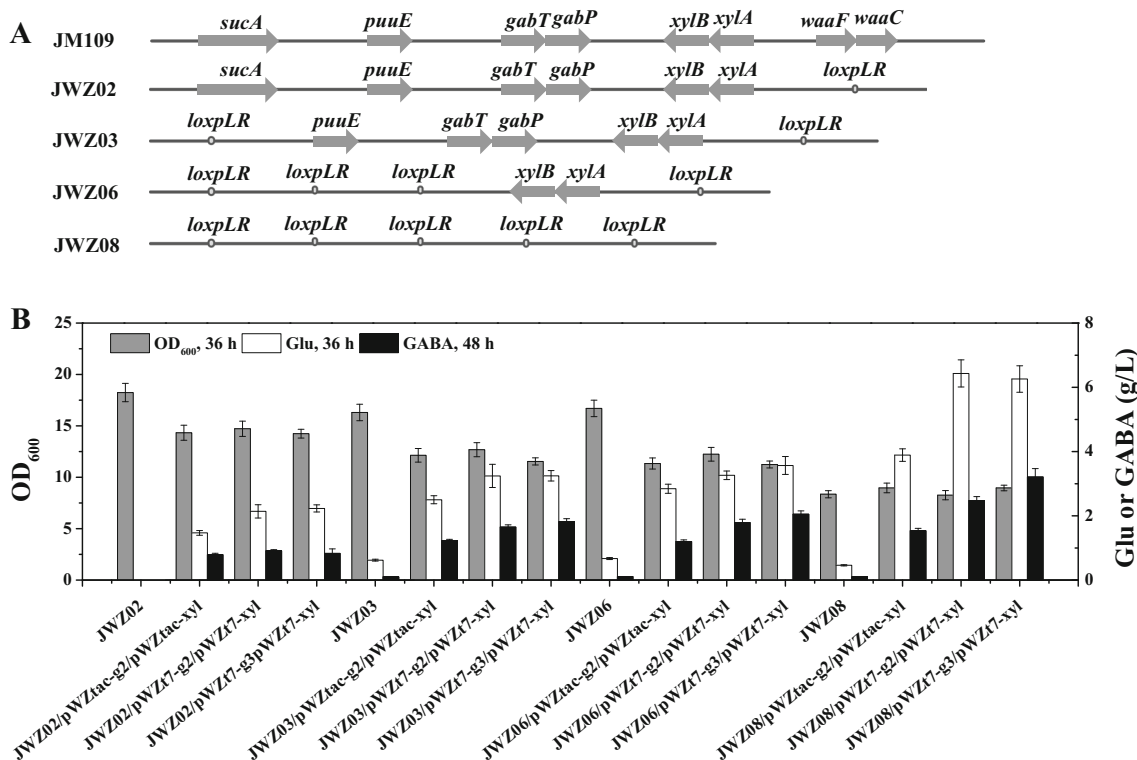

Fig. 5 **a** Comparison of different deletion mutants derived from *E. coli* W3110. **b** Glu production at 36 h, OD_{600} at 36 h, and GABA production at 48 h after two-stage pH control fermentation in *E. coli* strains JWZ2, JWZ2/pWZtac-g2/pWZt7-xyl, JWZ2/WZt7-g2/pWZt7-xyl, JWZ2/WZt7-g3/pWZt7-xyl, JWZ3, JWZ3/pWZtac-g2/pWZt7-xyl, JWZ3/WZt7-g2/pWZt7-xyl, JWZ3/WZt7-g3/pWZt7-xyl, JWZ6, JWZ6/pWZtac-g2/pWZt7-xyl, JWZ6/WZt7-g2/pWZt7-xyl, JWZ6/WZt7-g3/pWZt7-xyl, JWZ8, JWZ8/pWZtac-g2/pWZt7-xyl, JWZ8/WZt7-g2/pWZt7-xyl, and JWZ8/WZt7-g3/pWZt7-xyl

pWZt7-xyl, IPTG-controlled *lac* operators are behind the T7 promoters (T7-lac promoter) (Dubendorf and Studier 1991). So, even though the expression of T7 RNA polymerase is not affected by IPTG, IPTG is still required to activate the genes in pWZt7-g2, pWZt7-g3, and pWZt7-xyl. Effects of IPTG additions ranging from 0 to 2 mM were investigated, and 1 mM IPTG was found to be the best (Fig. 6a).

Medium volume in flask is related to dissolve oxygen, which is important in aerobic fermentation of *E. coli*. The effect of medium volume in 250-mL flask on the GABA production was investigated. Cell growth and Glu and GABA productions decreased with the increased of medium volume (Fig. 6a).

GadB is only active in acid conditions; thus, during the period for GABA production, pH is needed to be adjusted. After 36 h, pH was adjusted to 3, 3.8, and 4.6, and 1.06, 2.34, and 3.21 g/L GABA were produced at 48 h, respectively (Fig. 6a). Therefore, pH 4.6 was chosen to be the optimum pH.

XTB medium containing abundant yeast extract and peptone was used to produce GABA. Lowering the content of yeast extract and peptone in the medium would lower the cost for GABA production. As shown in Fig. 6b, the concentrations of peptone and yeast extract were optimized and the optimized concentrations of peptone and yeast extract are 2.4 and 24 g/L, respectively. Under the optimum conditions, 6.89 g/L Glu was produced at 36 h, and 3.81 g/L GABA was produced at 48 h (Fig. 6b).

JWZ08/pWZt7-g3/pWZt7-xyl was fermented under the optimum conditions. In the first stage for Glu accumulation, pH was controlled between 6.5 and 7.5, and 1 mM IPTG was added at 2 h when OD_{600} reached 1.0; in the second stage for GABA production, pH was adjusted to 4.6 and 10 mM PLP was added. As shown in Fig. 7, cells entered the stationary phase at 18 h when xylose was almost consumed. Glu production was continuously increased in the first 36 h and reached around 6.89 g/L at 36 h, and then slowly increased to 7.08 g/L at 48 h and 7.19 g/L at 60 h, respectively. After pH was adjusted to 4.6, Glu concentration decreased with the production of GABA. When pH was adjusted to 4.6 at 36, 48, and 60 h, 3.95, 3.57, and 2.75 g/L GABA was produced in JWZ08/pWZt7-g3/pWZt7-xyl at 60, 72, and 84 h, respectively (Fig. 7).

Discussion

Xylose, a key building block in most hemicelluloses, can be used by some bacteria via different pathways (Aristidou and Penttilä 2000), such as the xylose isomerase pathway, oxidoreductase pathway, and Weimberg pathway. For example, succinate (Liu et al. 2012b), ethanol (Saha et al. 2015), and lactate (Zhao et al. 2013) could be produced via the inherent xylose isomerase pathway; xylitol can be produced via oxido-

Fig. 6 GABA production in *E. coli* JWZ08/pWZt7-g3/pWZt7-xyl was optimized at temperature, media volume, pH, concentrations of IPTG, peptone, and yeast extract
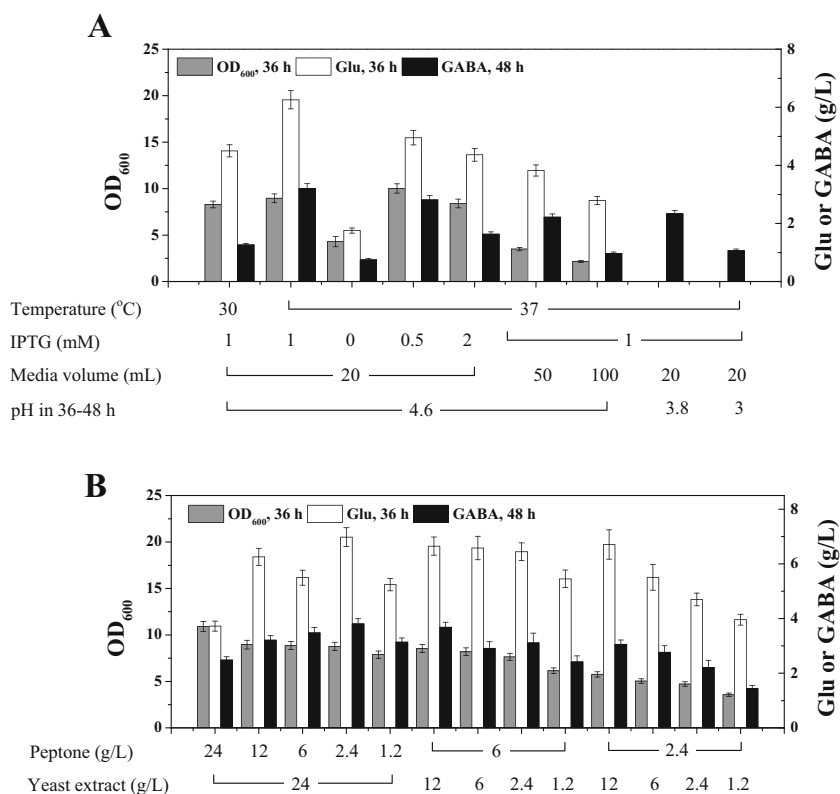
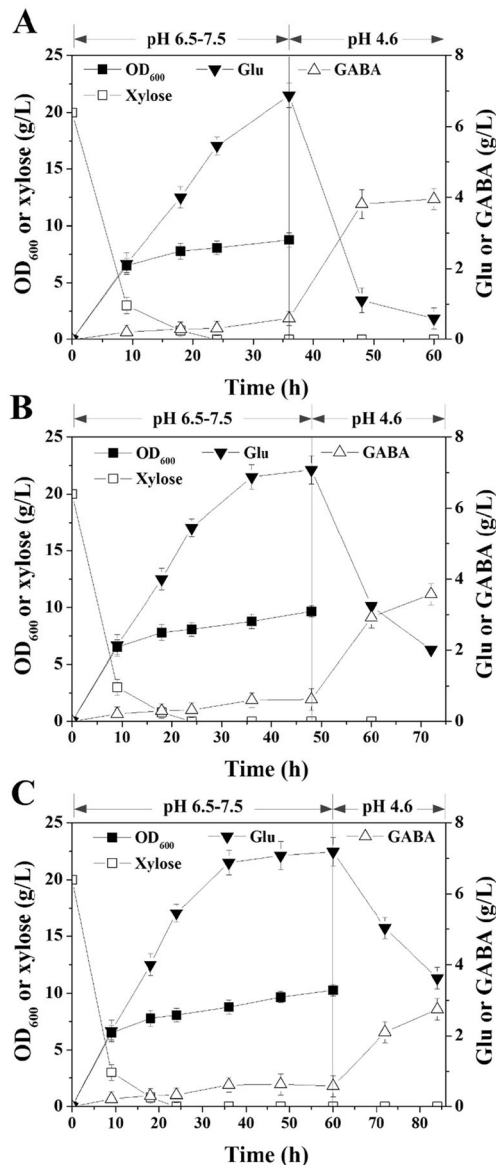**Fig. 7** Comparison of GABA production of *E. coli* JWZ08/pWZt7-g3/pWZt7-xyl when pH was adjusted to 4.6 at 36, 48, and 60 h, respectively

reductase pathway from *Candida* sp. and *Neurospora* sp. (Iverson et al. 2013; Su et al. 2015), xylonate (Cao et al. 2013), and 1,2,4-butanetriol (Valdehuesa et al. 2014) can be produced via the Weimberg pathway.

The first attempt to produce GABA from xylose in *E. coli* was conducted in this study. In *E. coli*, there is the xylose isomerase pathway which is too long to contribute the accumulation of GABA. The carbon flux from xylose to GABA requires only seven steps through Weimberg pathway but requires around 20 steps through the isomerase pathway. Through Weimberg pathway, xylose can be completely converted into α-ketoglutarate without carbon loss, while in the isomerase pathway, 17% carbon is lost in the form of CO_2 during TCA cycle (Radek et al. 2014). Furthermore, the isomerase pathway was long and meditated by some key

metabolites, such as glyceraldehyde-3-phosphate, pyruvate, and Acetyl-CoA. Therefore, Weimberg pathway which can covert xylose to α-ketoglutarate by five reaction steps, and the genes encoding Gdh and GadB which convert α-ketoglutarate to GABA were introduced into *E. coli* JM109 and W3110. GABA production was enhanced in both cases. Tac and T7 expression systems were used to express the genes. GABA productions in *E. coli* strains were often higher when the genes controlled by T7 expression system than by tac expression system. GABA productions in *E. coli* W3110 expressing the seven genes were slightly better than in JM109. However, when up to eight genes were deleted, *E. coli* mutants derived from JM109 produced more GABA than those derived from W3110 in the same condition, possibly because cell growth was significantly influenced in mutants derived from W3110. Deletion of *sucA*, *xylA*, and *xylB* significantly increased Glu and GABA productions in *E. coli*, but the cell growths of these mutants were slowed down. Compared with the wild-type *E. coli*, the growth rate of WWZ08/pWZt7-g2/pWZt7-xyl cells decreased fivefold, while the growth rate of JWZ08/pWZt7-g2/pWZt7-xyl decreased twofold. Plasmid pWZt7-g3 was constructed for extracellular expression of GadB facilitated by signal peptide TorA. Comparing with JWZ08/pWZt7-g2/pWZt7-xyl cells in which GadB was expressed intracellularly, more GABA was produced in JWZ08/pWZt7-g3/pWZt7-xyl cells.

Several studies focused on GABA production from glucose in *E. coli* have been reported (Pham et al. 2015; Pham et al. 2016a; Pham et al. 2016b), by expressing different genes, but GABA production never exceeded 1.3 g/L. In this study, up to 3.95 g/L GABA was produced from 20 g/L xylose in *E. coli* recombinant strain JWZ08/pWZt7-g3/pWZt7-xyl. Further studies were required to further improve GABA production from xylose in *E. coli*.

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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