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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Increasing L-threonine production in *Escherichia coli* by engineering the glyoxylate shunt and the L-threonine biosynthesis pathway

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Abstract

L-threonine is an important amino acid that can be added in food, medicine, or feed. Here, the influence of glyoxylate shunt on an L-threonine producing strain *Escherichia coli* TWF001 has been studied. The gene *iclR* was deleted, and the native promoter of the *aceBA* operon was replaced by the *trc* promoter in the chromosome of TWF001, the resulting strainTWF004 could produce 0.39 g L-threonine from1 g glucose after 36-h flask cultivation. Further replacing the native promoter of *aspC* by the *trc* promoter in the chromosome of TWF004 resulted in the strain TWF006. TWF006 could produce 0.42 g L-threonine from 1 g glucose after 36-h flask cultivation. Three key genes in the biosynthetic pathway of L-threonine, *thrA** (a mutated *thrA*), *thrB*, and *thrC* were overexpressed in TWF006, resulting the strain TWF006/pFW01-*thrA***BC*. TWF006/pFW01-*thrA***BC* could produce 0.49 g L-threonine from 1 g glucose after 36-h flask cultivation. Next, the genes *asd*, *rhtA*, *rhtC*, or *thrE* were inserted into the plasmid TWF006/pFW01-*thrA***BC*, and TWF006 was transformed with these plasmids, resulting the strains TWF006/pFW01-*thrA***BC-asd*, TWF006/pFW01-*thrA***BC-rhtA*, TWF006/pFW01-*thrA***BC-rhtC*, and TWF006/pFW01-*thrA***BC-thrE*, respectively. These four strains could produce more L-threonine than the control strain, and the highest yield was produced by TWF006/pFW01-*thrA***BC-asd*; after 36-h flask cultivation, TWF006/pFW01-*thrA***BC-asd* could produce 15.85 g/l L-threonine, i.e., 0.53 g L-threonine per 1 g glucose, which is a 70% increase relative to the control strain TWF001. The results suggested that the combined engineering of glyoxylate shunt and L-threonine biosynthesis pathway could significantly increase the L-threonine production in *E. coli*.

Keywords L-threonine production · *Escherichia coli* · Aspartate aminotransferase · Glyoxylate shunt

Introduction

L-threonine produced by microbial fermentation has an increasing market demand (Dong et al. 2012; Liu et al. 2015). A high yield of L-lysine or L-glutamic acid can be produced by *Corynebacterium glutamicum* (Hashimoto 2017), but not L-threonine (Dong et al. 2016); therefore, *Escherichia coli* became the important strain for L-threonine production (Dong et al. 2011; Lee et al. 2006). Starting from oxaloacetate, an intermediate of the Krebs cycle, the biosynthesis of L-threonine in *E. coli* consists of six enzymatic steps (Fig. 1). First, oxaloacetate is converted to L-aspartate by aspartate aminotransferase encoded by *aspC*. The second reaction is catalyzed by three aspartate kinases (I, II, III). Aspartate kinase I encoded by *thrA* can be inhibited by L-threonine, and its synthesis is repressed by L-threonine and L-isoleucine; the feedback inhibition can be removed by replacing the 1034th C with T in *thrA* (Lee et al. 2003). Aspartate kinase II is encoded by *metL*, while aspartate kinase III is encoded by *lysC*. Both aspartate kinases I and II have the second enzymic activity of homoserine dehydrogenase; thus, they also catalyze the fourth step (Fig. 1). Aspartate kinase I is the most abundant one among the three aspartate kinases in *E. coli*. The third reaction is catalyzed by aspartate semialdehyde dehydrogenase encoded by *asd*. The last two reactions are catalyzed by homoserine kinase encoded by *thrB* and threonine synthase encoded by *thrC*. In *E. coli*, *thrB* and *thrC* are clustered with

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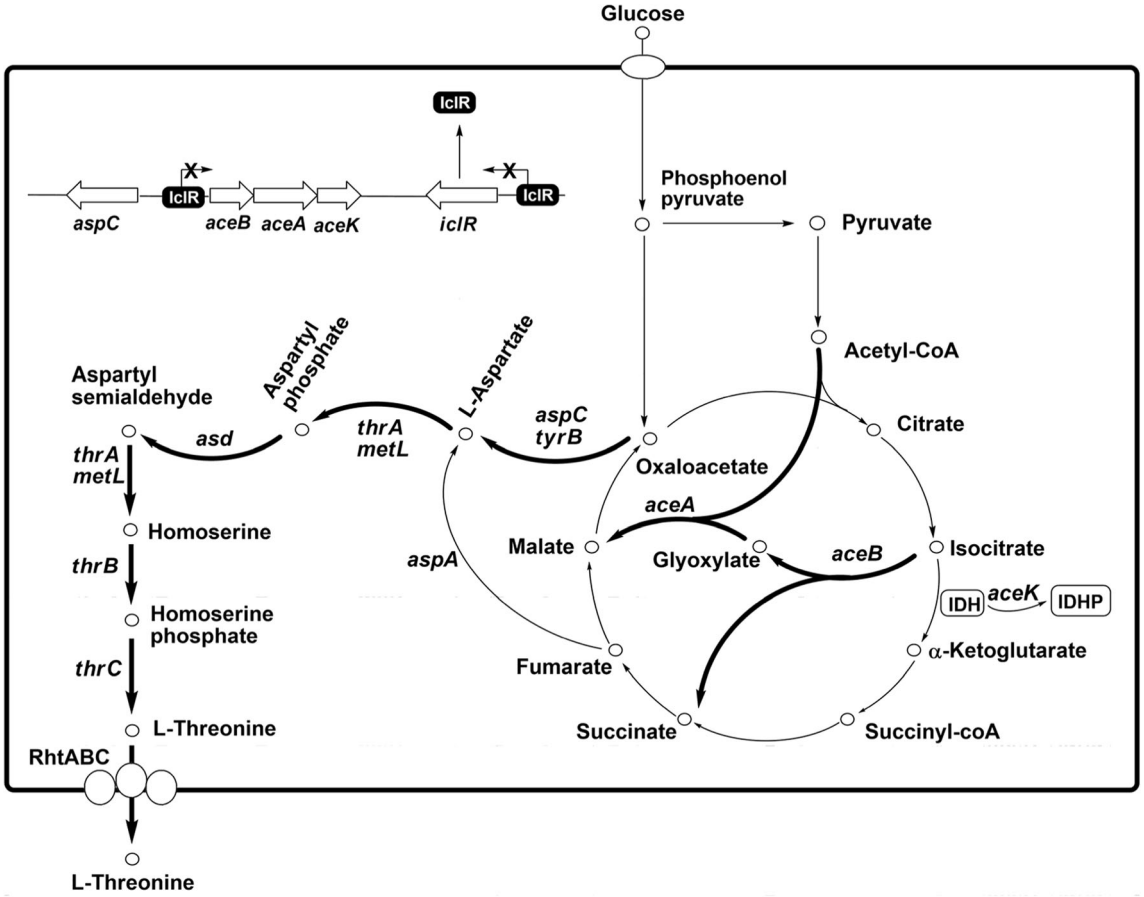

Fig. 1 The glyoxylate bypass and L-threonine biosynthetic pathway in *E. coli*. IclR, the transcription factor encoded by *iclR*, represses the *aceBAK* operon and *iclR*, by binding to the IclR-binding boxes at the upstream of the *aceBAK* operon and the upstream of *iclR*. The Krebs cycle and the glyoxylate bypass are mediated by AceK. In the complete Krebs cycle, isocitrate is converted to α-ketoglutarate by the enzyme isocitrate dehydrogenase (IDH). AceK encoded by *aceK* phosphorylates IDH, causing its inactivation. The inactivation of IDH increases the conversion of isocitrate to glyoxylate. Glyoxylate is then combined with acetyl-CoA to form malate, completing the glyoxylate bypass. RhtABC, different L-threonine export proteins

thrA in the *thrABC* operon. There are three export proteins for L-threonine in *E. coli* (Yuzbashev et al. 2013), which are encoded by *rhtA* (Livshits et al. 2003), *rhtB* (Zakataeva et al. 1999), and *rhtC* (Kruse et al. 2002), respectively. The expression of these three exporters can be induced at different conditions (Livshits et al. 2003). L-threonine export encoded by *thrE* in *C. glutamicum* could also efficiently function in *E. coli* (Kruse et al. 2002). Recently, the fourth L-threonine export encoded by *yecC* has been characterized in *E. coli* (Xu et al. 2017).

Production of L-threonine in *E. coli* can be enhanced by inactivating the transcription factor IclR encoded by *iclR* (Lee et al. 2007). IclR is a repressor for the *aceBAK* operon, which encodes isocitrate lyase (*aceB*), malate synthase (*aceA*), and isocitrate dehydrogenase kinase/phosphorylase (*aceK*) in the glyoxylate bypass (Fig. 1), it also represses the expression of *iclR* in an autogenous manner (Yamamoto and Ishihama 2003). There are two IclR-binding boxes at the upstream of the *aceBAK* operon and one IclR-binding box at the upstream of *iclR*. Regulator AceK determines the carbon flux distribution between the Krebs cycle and the glyoxylate bypass (Zheng et al. 2012). In the complete Krebs cycle, isocitrate dehydrogenase (IDH) converts isocitrate to α-ketoglutarate and carbon dioxide; when acetate becomes the sole carbon source, AceK inactivates IDH, increasing the conversion of isocitrate to glyoxylate (Fig. 1). Then, glyoxylate reacts with acetyl-CoA, producing malate through the glyoxylate bypass. When *E. coli* is grown in the stationary phase, glucose is usually completely consumed while acetate produced during the exponential phase becomes the major carbon source; therefore, the glyoxylate bypass is activated (Kumari et al. 2000).

In this study, a series of L-threonine producing *E. coli* mutant strains was constructed through the metabolic engineering, the glyoxylate bypass, and the biosynthetic pathway of L-threonine. One of the strains, TWF006/pFW01-*thrA***BC-asd*, could produce 15.85 g/l L-threonine after 36-h flask fermentation, which is a 70% increase relative to the control strain TWF001.

Materials and methods

Bacteria and culture conditions

Bacteria and plasmids used in this work are listed in Table 1. *E. coli* cells were grown at 37 or 30 °C in an LB medium (5 g/l yeast extract, 10 g/l tryptone, and 10 g/l NaCl) with 200 rpm shaking. Kanamycin (50 mg/l), spectinomycin (50 mg/l), triclosan (100 mg/l), isopropyl-D-thiogalactopyranoside (IPTG; 0.5 mM), or arabinose (10 mM) was added when necessary. L-threonine-producing *E. coli* strain TWF001 (CCTCC no. M2017730) was originally isolated from soil and is closely related to *E. coli* MG1655, based on their 16S rDNA sequences.

Table 1 Bacterial trains and plasmids used in this study

Strains or plasmids	Description	Sources
Strains		
JM109	Wild-type *E. coli*	NEB
MG1655	Wild-type *E. coli* K-12; $F^{-}\lambda^{-}$ rph-1	CGSC 6300
ATCC13032	Wild-type *C. glutamicum*	ATCC
TWF001	L-threonine-producing *E. coli* strain	This study
TWF001/pFW01	TWF001 harboring pFW01	This study
TWF001/pFW01-*thrA*BC*	TWF001 harboring pFW01-*thrA*BC*	This study
TWF002	TWF001 *PaceBA::Ptrc*	This study
TWF002/pFW01	TWF002 harboring pFW01	This study
TWF002/pFW01-*thrA*BC*	TWF002 harboring pFW01-*thrA*BC*	This study
TWF003	TWF001Δ*iclR*	This study
TWF003/pFW01	TWF003 harboring pFW01	This study
TWF003/pFW01-*thrA*BC*	TWF003 harboring pFW01-*thrA*BC*	This study
TWF004	TWF001Δ*iclR, PaceBA::Ptrc*	This study
TWF004/pFW01	TWF004 harboring pFW01	This study
TWF004/pFW01-*thrA*BC*	TWF004 harboring pFW01-*thrA*BC*	This study
TWF005	TWF001Δ*iclR, PaspC::Ptrc*	This study
TWF005/pFW01	TWF005 harboring pFW01	This study
TWF005/pFW01-*thrA*BC*	TWF005 harboring pFW01-*thrA*BC*	This study
TWF006	TWF001Δ*iclR, PaceBA::Ptrc, PaspC::Ptrc*	This study
TWF006/pFW01	TWF006 harboring pFW01	This study
TWF006/pFW01-*thrA*BC*	TWF006 harboring pFW01-*thrA*BC*	This study
TWF006/pFW01-*thrA*BC-asd*	TWF006 harboring pFW01-*thrA*BC-asd*	This study
TWF006/pFW01-*thrA*BC-rhtA*	TWF006 harboring pFW01-*thrA*BC-rhtA*	This study
TWF006/pFW01-*thrA*BC-rhtC*	TWF006 harboring pFW01-*thrA*BC-rhtC*	This study
TWF006/pFW01-*thrA*BC-thrE*	TWF006 harboring pFW01-*thrA*BC-thrE*	This study
Plasmids		
pTrc99A	Amp^{R}, *trc* promoter, cloning vector	(Amann et al. 1988)
pTargetF	*pMB1 aadA* sgRNA	(Jiang et al. 2015)
pTargetF-*iclR*	*pMB1 aadA* sgRNA-*iclR*	This study
pTargetF-*aceBA*	*pMB1 aadA* sgRNA-*aceBA*	This study
pTargetF-*aspC*	*pMB1 aadA* sgRNA-*aspC*	This study
pF2	Triclosan-resistant vector, *f1 ori*, 3.2-kb	(Jang and Magnuson 2013)
pFW01	Derived from pF2	This study
pFW01-*thrA*BC*	pFW01 containing *thrA*BC*	This study
pFW01-*thrA*BC-asd*	pFW01 containing *thrA*BC* and *asd*	This study
pFW01-*thrA*BC-rhtA*	pFW01 containing *thrA*BC* and *rhtA*	This study
pFW01-*thrA*BC-rhtC*	pFW01 containing *thrA*BC* and *rhtC*	This study
pFW01-*thrA*BC-thrE*	pFW01 containing *thrA*BC* and *thrE*	This study

Construction of *E. coli* mutant strains TWF002, TWF003, TWF004, TWF005, and TWF006

The primers used in this work are listed in Table 2. Most primer sequences were designed according to the genome sequence of *E. coli* MG1655. Genomic DNA of *E. coli* TWF001 was isolated and used as templates for PCR amplification of genes or their upstream and downstream fragments. Plasmids and genomic DNA were extracted by using the TIANGEN prep kit (Tiangen, Beijing, China) according to the manufacturer's instruction. PrimeSTAR HS DNA Polymerase (Takara, Dalian, China) was used for PCR. Restriction endonucleases and T4 DNA ligase were purchased from Thermo Scientific (Waltham, USA). Both the in-frame gene deletion and the promoter replacement in the chromosome of *E. coli* were carried out according to the published method (Jiang et al. 2015).

TWF002 was constructed from TWF001 by replacing the two IclR-binding boxes and the native promoter of the *aceBAK* operon with the *trc* promoter (Fig. 2a). The upstream fragment of *aceBA* gene was amplified using the primer pair *aceBA*f1 and *aceBA*r1. The downstream fragment of the *aceBA* gene was amplified using the primer pair *aceBA*f3 and *aceBA*r3. The *trc* promoter was amplified from pTrc99A (Amann et al. 1988) using the primers *aceBA*f2 and *aceBA*r2. These three PCR products were overlapped together by using the primers *aceBA*f1 and *aceBA*r3, resulting in the replacement fragment P*trc-aceBA*. The pTargetF-*aceBA* was obtained from pTargetF (Jiang et al. 2015) by inverse PCR using the primers sgRNA-*aceBA*-F and T-sgRNA-R, following by self-ligation, and was confirmed by using primers T-*aceBA*-F and T-sgRNA-R. The plasmid pCas (Jiang et al. 2015) was transformed into TWF001, and the competent TWF001/pCas cells were prepared as described previously (Sharan et al. 2009). Arabinose was added to the culture when preparing the competent TWF001/pCas cells to activate the Red enzymes for recombination. Then, 200 ng pTargetF-*aceBA* and 500 ng P*trc-aceBA* were mixed with 80 μl TWF001/pCas competent cells and electroporated. Cells were recovered at 30 °C for 2 h, then spread onto LB agar plates containing kanamycin and spectinomycin, and incubated for 48 h at 30 °C. Transformants were identified by colony PCR using the primers *aceBA*f1 and *aceBA*r2 and confirmed by DNA sequencing of the primer region of *aceBA*. At the end, pTargetF-*aceBA* was cured by adding IPTG, and pCas was cured by growing at 42 °C overnight.

TWF003 was constructed from TWF001 by deletion of *iclR* (Fig. 2a). The upstream fragment of *iclR* was amplified using the primer pair *iclR*f1 and *iclR*r1. The downstream fragment of *iclR* was amplified using the primers*iclR*f2 and *iclR*r2. These PCR products were linked together by overlapping PCR using the primers*iclR*f1 and *iclR*r2. The pTargetF-*iclR* was obtained from pTargetF by inverse PCR using the primers sgRNA-*iclR*-F and T-sgRNA-R, following by self-ligation, and was confirmed by using the primers T-*iclR*-F and T-sgRNA-R. Then, 200 ng pTargetF-*iclR* and 500 ng of the deletion fragment were mixed with 80-μl TWF001/pCas competent cells and electroporated. Cells were recovered, and the deletion mutant TWF003 was selected on LB plates containing kanamycin and spectinomycin. The correct *iclR* deletion in TWF003 was identified by PCR using the primers *iclR*f1 and *iclR*r2. At the end, pTargetF-*iclR* was cured by adding IPTG, and pCas was cured by growing at 42 °C overnight.

TWF004 was constructed from TWF003 by replacing the IclR-binding boxes and the native promoter of the *aceBAK* operon with the *trc* promoter, using the same method and primer pairs used for the construction of TWF002 (Fig. 2a).

TWF005 was constructed from TWF003 by inserting the *trc* promoter in the upstream of *aspC* (Fig. 2a). The upstream fragment of the *aspC* gene was amplified using the primers *aspC*f1 and *aspC*r1. The downstream fragment of the *aspC*gene was amplified using the primers *aspC*f3 and *aspC*r3. The *trc* promoter was amplified from pTrc99A using the primers *aspC*f2 and*aspC*r2. These three PCR products were overlapped together by using the primers *aspC*f1 and *aspC*r3, resulting in the replacement fragment P*trc-aspC*. The pTargetF-*aspC* was obtained from pTargetF by inverse PCR using the primers sgRNA-*aspC*-F and T-sgRNA-R, following by self-ligation, and was confirmed by using primers T-*aspC*-F and T-sgRNA-R. Arabinose was added to the culture when preparing the competent TWF003/pCas cells to activate the Red enzymes for recombination. Then, 200 ng pTargetF-*aspC* and 500 ng P*trc-aspC* were mixed with 80-μl TWF003/pCas competent cells and electroporated. Transformants were identified by colony PCR using the primers *aspC*f1 and *aspC*r2 and confirmed by DNA sequencing of the promoter region of *aspC*. At the end, pTargetF-*aspC* was cured by adding IPTG, and pCas was cured by growing at 42 °C overnight.

TWF006 was constructed from TWF004 by inserting the *trc* promoter in the upstream of *aspC*, using the same method and primer pairs used for the construction of TWF005 (Fig. 2a). The in-frame gene deletion and/or the promoter replacement in the chromosome of the above *E. coli* mutants was confirmed by DNA sequencing.

Construction of different plasmids

The vector pFW01 was constructed from pF2 (Jang and Magnuson 2013) by inserting the PJ23101 (5'-TTTACAGC TAGCTCAGTCCTAGGTATTATGCTAGC-3') promoter (http://partsregistry.org/Part:BBa_J23101) and replacing *mfabI* with *Pseudomonas aeruginosa fabV* (Zhu et al. 2010). To insert the PJ promoter in pF2, a large linear DNA fragment was amplified with primers PJ-F and PJ-R using pF2 as a template and then self-ligated. The correct insertion of the PJ

Table 2 Primers used in this study

Primer name	Sequence (5′-3′)	Restriction site
sgRNA-*iclR*-F	ACGATGAGGAACATGCACTGGTTTTAGAGCTAGAAATAGC	
T-*iclR*-F	ACGATGAGGAACATGCACTG	
*iclR*f1	CTTGTTGCTAAAGATATGACG	
*iclR*r1	CAAACCATACTGGCATAAACGCAGAGGCAATATTCTGCCCATC	
*iclR*f2	GATGGGCAGAATATTGCCTCTGCGTTTATGCCAGTATGGTTTG	
*iclR*r2	GATCAGATCCGCGCCACCTTC	
sgRNA-*aceBA*-F	AACTTCCCCACTGTGAACGAGTTTTAGAGCTAGAAATAGC	
T-*aceBA*-F	AACTTCCCCACTGTGAACGA	
*aceBA*f1	ATTCAGGATCAGAACTTTGAC	
*aceBA*r1	GTCAAGGTATATCTCCTTCTAGGTGCTGAATCGCTTAACG	
*aceBA*f2	CGTTAAGCGATTCAGCACCTAGAAGGAGATATACCTTGAC	
*aceBA*r2	CATCGTGCAGCTCCTCGTCATGGGTCTGTTTCCTGTGTGAAAT	
*aceBA*f3	ATTTCACACAGGAAACAGACCCATGACGAGGAGCTGCACGATG	
*aceBA*r3	CAAACAGGCTGCCGGGGATTG	
sgRNA-*aspC*-F	TTGTTAAATGCCGAAAAAACGTTTTAGAGCTAGAAATAGC	
T-*aspC*-F	ACTAGTATTATACCTAGGACTGAGC	
*aspC*f1	GTTAATCAGTGCATCGAAGTC	
*aspC*r1	CAATTTCACACAGGAAACAGACCGTTTTCATCAGTAATAGTTGG	
*aspC*f2	CCAACTATTACTGATGAAAACGGTCTGTTTCCTGTGTGAAATTG	
*aspC*f2	GTTTACCAGTTCTAATAGCACAGAAGGAGATATACCTTGAC	
*aspC*r3	GTCAAGGTATATCTCCTTCTGTGCTATTAGAACTGGTAAAC	
*aspC*r3	GGCTTTGGTATCGTTGGTGC	
T-sgRNA-R	ACTAGTATTATACCTAGGACTGAGC	
PJ-F	TTTACAGCTAGCTCAGTCCTAGGTATTATGCTAGCAAAATTAAG AGGTATATACATATGACCATGATTACGCCAAGC	
PJ-R	CCTAGGACTGAGCTAGCTGTAAAGCCTGGGGTGCCTAATG	
pJF-F	ACTCTTCCTTTTTCAATATTATTGAAGC	
pJF-R	CTGTCAGACCAAGTTTACTCATATATACTT	
fabV-F	CTTCAATAATATTGAAAAAGGAAGAGTATGATCATCAAACCGCGCG	
fabV-R	TATATGAGTAAACTTGGTCTGACAGTCAGGCCTGGATCAGGTTGG	
*thrA***BC*-U-F	CGGGATCC**AGAAGGAGATATACC**ATGCGAGTGTTGAAGTTCGG	*Bam*HI
*thrA***BC*-U-R	GCGCGTGACATCGCTGCAAAGACGCGCGCCGCCATGCCGACCATCCCTTTCAT	
*thrA***BC*-D-F	TGGCGGCGCGCGTCTTTGCAGCGATGTCACGCGCCCGTATTTTCGTGGTGCT	
*thrA***BC*-D-R	CTCTAGATTACTGATGATTCATCATCAATTT	*Xba*I
asd-F	GCTCTAGA**AGAAGGAGATATACC**TTACGCCAGTTGACGAAGCAT	*Xba*I
asd-R	CGAGCTCATGTTGGTTTTATCGGCTGGCGCGG	*Sac*I
rhtA-F	GCTCTAGA**AGAAGGAGATATACC**TTAATTAATGTCTAATTC	*Xba*I
rhtA-R	CGAGCTCATGCCTGGTTCATTACGTAAA	*Sac*I
rhtC-F	GCTCTAGA**AGAAGGAGATATACC**ATGCGAGTGTTGAAGTTCGG	*Xba*I
rhtC-R	CGAGCTCTCACCGCGAAATAATCAAATG	*Sac*I
thrE-F	GCTCTAGA**AGAAGGAGATATACC**ATGTTGAGTTTTGCGACCCTTCGTG	*Xba*I
thrE-R	CGAGCTCTTACCTTTTATTACCGAATCTCTGATT	*Sac*I
RT-16sRNA-F	TCGGGAACCGTGAGACAGG	
RT-16sRNA-R	CCGCTGGCAACAAAGGATAAG	
RT-*thrA*-F	TTCCTACTTCGGCGCTAAAG	
RT-*thrA*-R	TTGCCATGTTATTCAGATTG	
RT-*thrB*-F	GCTGAACCCTACCGTGAACG	
RT-*thrB*-R	ATATGAACAAAACCTTCCTG	
RT-*thrC*-F	CGCTGGCATTTAAAGATTTC	

Table 2 (continued)

Primer name	Sequence (5′-3′)	Restriction site
RT-*thrC*-R	TTGCCGCCCAATGTACAGAAC	
RT-*rhtA*-F	GTGTCAGTGTTTCTCCGAG	
RT-*rhtA*-R	GGCACTGGTCGGTTATTCC	
RT-*rhtC*-F	AAATATGAAAATTGGAACTG	
RT-*rhtC*-R	TTTTCCACAACCTCACCGAG	
RT-*aceB*-F	GTCCCTATTTCTATCTGCCG	
RT-*aceB*-R	TCCCAACGACCGCAGTTCAG	
RT-*aceA*-F	GTCCTGAATGCCTTTGAAC	
RT-*aceA*-R	TCGCTGTCATACGGGTCGC	
RT-*aspC*-F	ACCGCATATACGCCAAACTC	
RT-*aspC*-R	GTTGCCACCATCCTGAGC	
RT-*aspA*-F	GGAAGAGTAAACTGCGATAC	
RT-*aspA*-R	GATGAAGTCCTGAACAACGG	
RT-*tyrB*-F	CAAACCCTTGGCGGCTCCGG	
RT-*tyrB*-R	TTAGTCGCTTCGTCATACC	
RT-*gltA*-F	GCGACCGATTCTAACTACC	
RT-*gltA*-R	CCACACATGACTGCCATTG	
RT-*icdA*-F	TTATCTTCCGTGAAAACTC	
RT-*icdA*-R	AGTCACAGAGTCACGATCG	

The restriction enzyme sites are underlined; the SD sequences are bold

promoter in pF2 was confirmed by DNA sequencing. Using this new plasmid as a template, a linear DNA fragment was amplified with primers pJF-F and pJF-R; meanwhile, another DNA fragment was amplified with primers *fabV*-F and *fabV*-R using the genomic DNA of *P. aeruginosa* PAO1 as a template. These two PCR products were ligated by using the ClonExpress II One Step Cloning Kit C112-01 (Vazyme, Nanjing, China), resulting the plasmid pFW01 (Fig. 2b).

Three key genes for L-threonine biosynthesis in *E. coli* were inserted in pFW01, resulting in the plasmid pFW01-*thrA***BC*. In *E. coli*, *thrA*, *thrB*, and *thrC* are clustered in the *thrABC* operon. The *thrA** indicates that the 1034th C in *thrA* is replaced with T. To create this point mutation, two DNA fragments were amplified by using primer pairs of *thrA***BC*-U-F/*thrA***BC*-U-R and *thrA***BC*-D-F/*thrA***BC*-D-R, respectively, using the genomic DNA of *E. coli* MG1655 as a template; then, they were ligated together by overlapping PCR using primers *thrA***BC*-U-F and *thrA***BC*-D-R, resulting the *thrA***BC* fragment. Next, the *thrA***BC* fragment was digested with *Bam*HI and *Xba*I, and pFW01 was digested with *Bam*HI and *Sac*I; then, the two fragments were ligated together, resulting the plasmid pFW01-*thrA***BC* (Fig. 2b). The correct sequence in pFW01-*thrA***BC* was checked by using colony PCR, restriction enzyme digestion, and DNA sequencing.

The plasmids pFW01 and pFW01-*thrA***BC* were transformed into different *E. coli* strains, forming recombinant strains TWF001/pFW01, TWF001/pFW01-*thrA***BC*, TWF002/pFW01, TWF002/pFW01-*thrA***BC*, TWF003/pFW01, TWF003/pFW01-*thrA***BC*, TWF004/pFW01, TWF004/pFW01-*thrA***BC*, TWF005/pFW01, TWF005/pFW01-*thrA***BC*, TWF005/pFW01, and TWF005/pFW01-*thrA***BC*.

Then, *asd*, *rhtA*, *rhtC*, and *thrE* were separately inserted in pFW01-*thrA***BC*, resulting pFW01-*thrA***BC-asd*, pFW01-*thrA***BC-rhtA*, pFW01-*thrA***BC-rhtC*, and pFW01-*thrA***BC-thrE*. Using the genomic DNA of *E. coli* MG1655 as a template, the *asd* fragment was amplified by using primers *asd*-F and *asd*-R, the *rhtA* fragment was amplified by using primers *rhtA*-F and *rhtA*-R, the *rhtC* fragment was amplified by using primers *rhtC*-F and *rhtC*-R. The *thrE* fragment was amplified by using primers *thrE*-F and *thrE*-R and the genomic DNA of *C. glutamicum* ATCC13032 as a template. Next, the *asd*, *rhtA*, *rhtC*, *thrE* fragments, and the plasmid pFW01-*thrA***BC* were digested with *Xba*I and *Sac* I; then, the four fragments were separately ligated with pFW01-*thrA***BC*, resulting the plasmid pFW01-*thrA***BC-asd*, pFW01-*thrA***BC-rhtA*, pFW01-*thrA***BC-rhtC*, and pFW01-*thrA***BC-thrE* (Fig. 2b). The four different plasmids were transformed into TWF006, resulting in the recombinant strains TWF006/pFW01-*thrA***BC-asd*, TWF006/pFW01-*thrA***BC-rhtA*, TWF006/pFW01-*thrA***BC-rhtC*, and TWF006/pFW01-*thrA***BC-thrE*.

Quantification of mRNA using real-time PCR

Real-time PCR (RT-PCR) was used to quantify mRNA levels of *aceA*, *aceB*, *thrA*, *thrB*, *thrC*, *rhtA*, *rhtB*, *rhtC*, *aspA*, *aspC*,

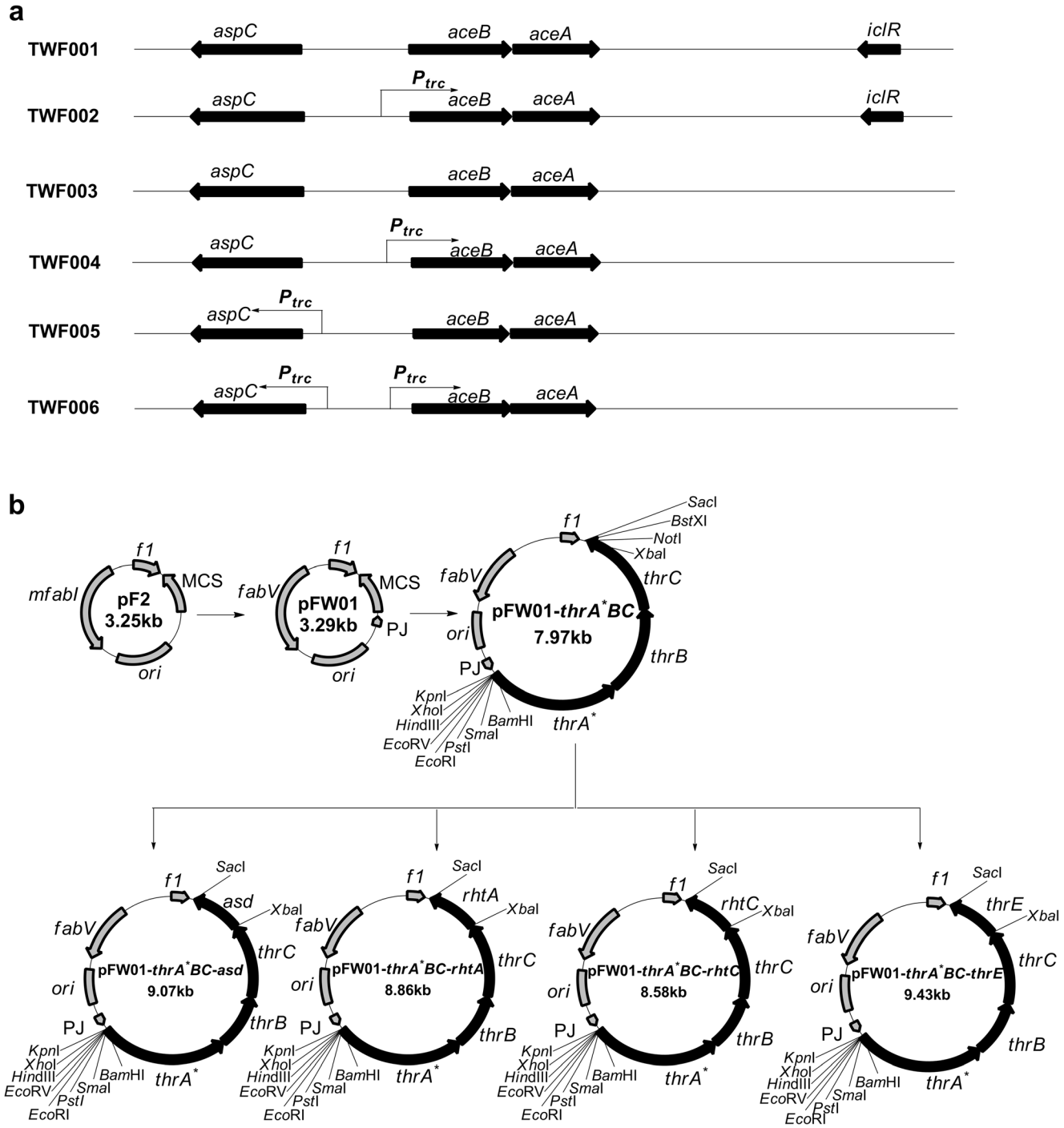

Fig. 2 **a** Genetic comparison of different *E. coli* strains constructed in this study. **b** Maps of different vectors constructed in this study

tyrB, *icdA*, and *gltA* in different *E. coli* strains. Total RNA was extracted from different *E. coli* cells grown at the mid-exponential phase using an RNA extraction kit (Bio Flux, Beijing, China). Residual DNA was removed from the RNA sample by DNaseI. The quality and amount of RNA were judged and quantified by electrophoresis. Using random hexamer primers, 500-ng RNA was reversely transcribed into cDNA using a Revert Aid™ First Strand cDNA synthesis kit (Fermentas, Shanghai, China). RT-PCR was performed using an ABI Step One RT-PCR system (Applied Biosystems, San Mateo, CA, USA) with a Real Master Mix kit (Tiangen, Beijing, China). Primers for detection of various genes are listed in Table 2. The following RT-PCR procedure was used:1 min at 94 °C, 40 cycles of 10 s at 94 °C, 30 s at 55 °C, and 15 s at 68 °C. The relative abundance of the targeted mRNAs was quantified based on the cycle

threshold value, which is defined as the number of cycles required to obtain a fluorescent signal above the background and was calculated according to the published method (Livak and Schmittgen 2001; Nolden et al. 2001). To standardize the results, the relative abundance of 16S rRNA was used as an internal standard control. All assays were performed in triplicate.

Flask fermentation

The threonine-producing *E. coli* strains were grown on LB plates for 24 h and transferred to a test tube containing 5 ml LB medium. After growing for 4 h at 37 °C, the OD_{600} of the culture was measured. Then, the culture was transferred to a 250-ml flask containing 25 ml LB medium, with the initial OD_{600} of 0.1. After growing for 4 h at 37 °C, 5-ml culture was transferred to 30-ml fermentation medium (2 g/l yeast extract, 2 g/l citric acid, 25 g/l $(NH4)_2SO_4$, 7.46 g/l KH_2PO_4, 30 g/l glucose, 2 g/l $MgSO_4 \cdot 7\ H_2O$, 5 mg/l $FeSO_4 \cdot 7H_2O$, 5 mg/l $MnSO_4 \cdot 4\ H_2O$, and 20 g/l $CaCO_3$, pH 6.8) (Lee et al. 2009). The fermentation was run for 36 h at 37 °C, with vigorous agitation (200 rpm).

Analytical procedures

Biomass was determined by measuring OD_{600} with UV-1800 spectrophotometer (Shimadzu, Japan). During fermentation, 1 ml of the culture broth was taken at different time points and centrifuged at 12,000 rpm for 5 min. The supernatant was used to analyze the levels of glucose and amino acids. The glucose concentration was measured with a SBA-40C biosensor (Institute of Biology, Shan-dong Academy of Science, China). To determine the levels of amino acids, the supernatant was diluted 20-folds, filtered, and analyzed by the 1200 series HPLC system (Agilent Technology, USA); the separation and quantification of amino acids were performed on a Thermo ODS-2HYPERSIL C18 column (250 mm × 4.0 mm, USA) by using the orthophthalaldehyde precolumn derivatization method (Kőrös et al. 2008). All the solvents used in HPLC analysis were purchased from Sigma-Aldrich (Shanghai, China).

Results

Modifying the glyoxylate shunt to increase L-threonine production in *E. coli* TWF001

E. coli TWF001 can produce 9.32 g/l L-threonine from 30 g/l glucose after 36-h flask fermentation when its OD_{600} reached the maximum (15.94) and glucose was completely consumed (Fig. 3). To further increase L-threonine in TWF001, the two IclR-binding boxes and the native promoter of the *aceBAK* operon were replaced by the *trc* promoter, resulted in the strain TWF002 (Fig. 2). To our surprise, L-threonine production in TWF002 dramatically decreased, comparing to the control TWF001 (Fig. 3c). TWF002 cells grew faster than TWF001 cells, reaching the maximum OD_{600} 17.94 after 36 h (Fig. 3a), and they also consumed more glucose than TWF001 cells at the same time point (Fig. 3b). This suggests that the glucose is consumed mainly for the growth rather than the production of L-threonine in TWF002.

To understand the decrease of L-threonine production in TWF002, the transcriptional levels of *aceA*, *aceB*, *thrA*, *thrB*, *thrC*, *rhtA*, *rhtB*, *rhtC*, *aspA*, *aspC*, *tyrB*, *icdA*, and *gltA* were determined by RT-PCR. Although the transcriptional levels of *aceA* and *aceB* were significantly increased as expected in TWF002, while *aspA*, *aspC*, *icdA*, and *tyrB* were downregulated (Fig. 3d). The gene *aspA* encodes L-aspartase that catalyzes the reaction from fumarate to L-aspartate, while the genes *tyrB* and *aspC* encode the aromatic aminotransferase and aspartate aminotransferase, respectively, and both enzymes can catalyze the reaction from oxaloacetate to L-aspartate (Fig. 1). The downregulation of these three genes might explain why L-threonine production in TWF002 decreased, considering L-aspartate is the key precursor for L-threonine biosynthesis. The transcriptional levels of genes relevant to the biosynthesis pathway and transport of L-threonine (*thrA*, *thrB*, *thrC*, *rhtA*, *rhtB*, *rhtC*, and *gltA*) in TWF002 were the same to the control TWF001 (Data not shown), further confirming that L-aspartate shortage is the main reason for the decreased L-threonine production in TWF002. In addition, the glutamate (2.25 g/l) was significantly accumulated in TWF002, suggesting that the tricarboxylic acid cycle is enhanced in TWF002 since glutamate is mainly synthesized from α-ketoglutarate, an intermediate metabolite in the tricarboxylic acid cycle. This is consistent with the better growth of TWF002. This experiment demonstrates that replacing the IclR-binding boxes and the native promoter of the *aceBAK* operon with the *trc* promoter did not enhance the L-threonine biosynthesis but improved the cell growth by enhancing the tricarboxylic acid cycle.

Since IclR represses the transcription of the *aceBAK* operon in *E. coli*, the *iclR* gene was deleted inTWF001, resulting in the strain TWF003 (Fig. 2). After 36-h flask fermentation, glucose was completely consumed, 11.76 g/l L-threonine were produced from 30 g/l glucose in TWF003, which is a 26% increase compared to the control TWF001 (Fig. 3). The transcriptional levels of *aceA* and *aceB* in TWF003 were significantly increased, and *icdA*, *aspA*, and *tyrB* were also upregulated, but *aspC* was downregulated (Fig. 3d). The two IclR-binding boxes and the native promoter of the *aceBAK* operon in TWF003 were replaced with the *trc* promoter, resulting the strain TWF004 (Fig. 2a). L-threonine production in TWF004 was on the same level to TWF003 (Fig. 3c). The transcriptional levels of *aceA* and *aceB* in TWF004 were much higher

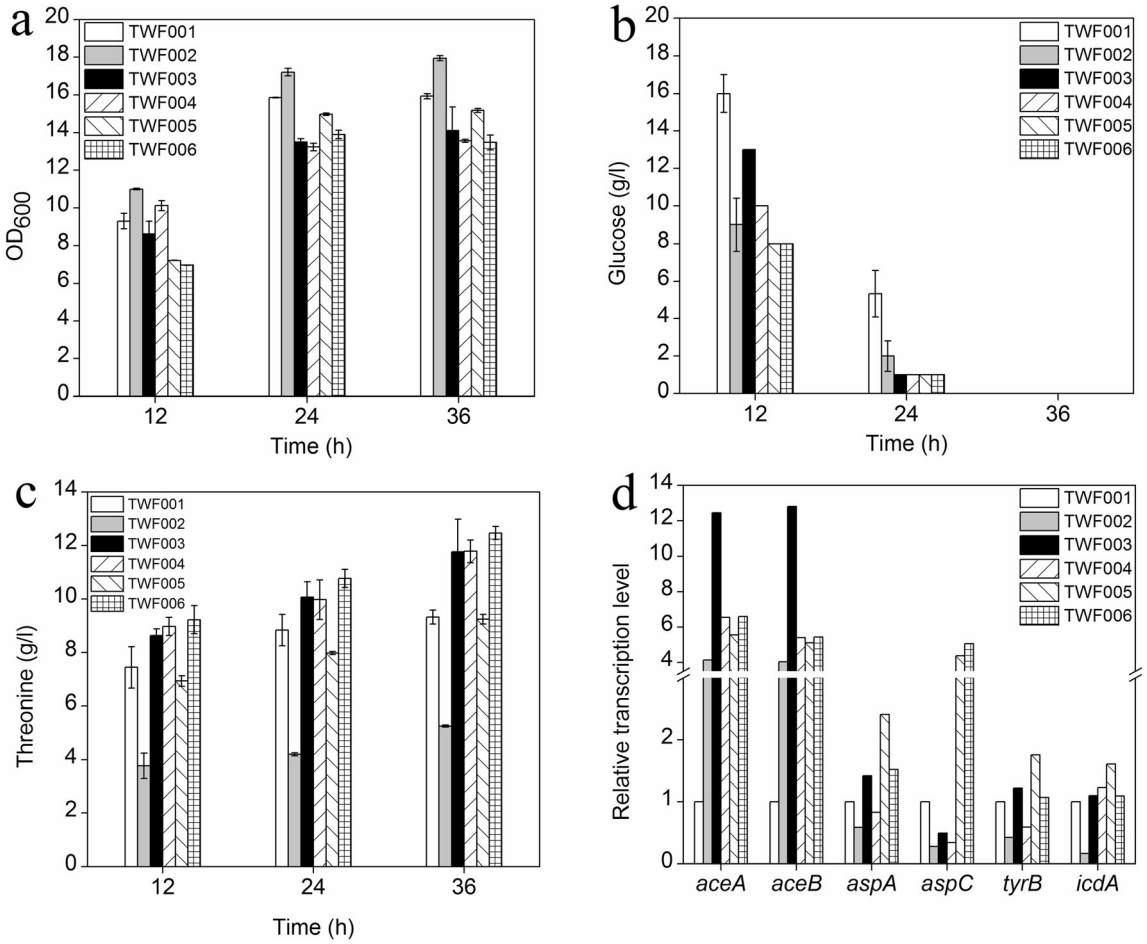

Fig. 3 Flask cultivation for L-threonine production in different *E. coli* strains TWF001, TWF002, TWF003, TWF004, TWF005, and TWF006. **a** Cell growth. **b** Glucose consumption. **c** L-threonine production. **d** Relative transcription levels of *aceA*, *aceB*, *aspA*, *aspC*, *tyrB*, and *icdA* analyzed by RT-PCR. The error bars indicate the standard deviations from three independent experiments

than that in TWF001 but lower than in TWF003 (Fig. 3d). This indicates that the native promoter of the *aceBAK* operon in *E. coli* might be stronger than the *trc* promoter, considering that *iclR* has been deleted in both TWF003 and TWF004, and the only difference is the native promoter of the *aceBAK* operon in TWF003 has been replaced by the *trc* promoter in TWF004. Interestingly, compared to the control TWF001, the transcriptional levels of *aspA*, *aspC*, and *tyrB* were downregulated (Fig. 3d) even though the L-threonine production in TWF004 was increased.

Enhancing the expression of aspartate aminotransferase to increase L-threonine production in *E. coli*

The gene *aspC* encodes aspartate aminotransferase that catalyzes the reaction from oxaloacetate to L-aspartate, but its transcriptional levels were downregulated in TWF003 and TWF004. To draw the carbon flow from Krebs cycle to L-threonine biosynthesis, the *trc* promoter was inserted in the upstream of *aspC* inTWF003 and TWF004, resulting in TWF005 and TWF006, respectively (Fig. 2). Although L-threonine production in TWF005 was similar to that in TWF001, the cell growth of TWF005 is much better than of TWF003 (Fig. 3). Compared to the control TWF001, the transcriptional levels of *aceA*, *aceB*, and *aspC* were significantly increased in TWF005, and *aspA*, *tyrB*, and *icdA* were also upregulated (Fig. 3d). TWF006 produced 12.47 g/l L-threonine from 30 g/l glucose after 36-h flask fermentation (Fig. 3c), which is a 34% increase compared to the control TWF001. OD_{600} of TWF006 reached the maximum (13.9) at 24 h, but the L-threonine production continued to increase until 36 h when glucose was completely consumed (Fig. 3). The transcriptional levels of *aceA*, *aceB*, and *aspC* were significantly increased in TWF006, *aspA* and *tyrB* were also upregulated (Fig. 3d). This indicates that the carbon flux in TWF006 was efficiently directed to the L-threonine biosynthesis pathway.

Overexpressing the key enzymes in L-threonine biosynthesis pathway to further increase L-threonine production

Based on RT-PCR analysis, the transcriptional levels of the three key genes in L-threonine biosynthesis pathway, *thrA*, *thrB*, and *thrC*, in TWF002, TWF003, TWF004, TWF005, and TWF006, were the same to the control TWF001 (Data not shown). Therefore, the three genes *thrA*, *thrB*, and *thrC* were overexpressed in these *E. coli* strains to further increase L-threonine production (Fig. 4). The three genes were inserted in the vector pFW01 which was constructed by replacing the *mfabI* gene in the plasmid pF2 (Jang and Magnuson 2013) with the *fabV* gene (Zhu et al. 2010). Not like other plasmids which need addition of antibiotics to maintain in *E. coli* cells, pFW01 can maintain in *E. coli* by adding a tiny amount of triclosan. Triclosan specifically inhibits fatty acid synthesis in *E. coli* by inactivating an enoyl-acyl carrier protein reductase encoded by *fabI*. The pF2 contains a mutant *fabI* (*mfabI*) (Jang and Magnuson 2013), while pFW01 contains the gene *fabV* from *Pseudomonas aeruginosa* PAO1 (Fig. 2); *fabV* encodes a triclosan-resistant enoyl-ACP reductase in *P. aeruginosa* which is more sensitive to triclosan than that encoded by *mfabI* (Zhu et al. 2010). To enhance the expression of the genes cloned into the MCS, the promoter pJ has been inserted on the upstream of MCS in pFW01 (Fig. 2). *E. coli* harboring pFW01 grows much better than *E. coli* harboring pF2 in the presence of the same amount of triclosan (data not shown). Three key genes *thrA**, *thrB*, and *thrC* in the L-threonine biosynthetic pathway in *E. coli* were inserted in pFW01, resulting pFW01-*thrA***BC* (Fig. 2). The *thrA** is a *thrA* mutant in which the 1034th C has been replaced with T, and it encodes a mutant of aspartate kinase I which is resistant to feedback inhibition (Lee et al. 2003). The plasmids pFW01 and pFW01-*thrA***BC* were transformed into different *E. coli* strains, resulting in TWF001/pFW01, TWF001/pFW01-*thrA***BC*, TWF002/pFW01, TWF002/pFW01-*thrA***BC*, TWF003/pFW01, TWF003/pFW01-*thrA***BC*, TWF004/pFW01, TWF004/pFW01-*thrA***BC*, TWF005/pFW01, TWF005/pFW01-

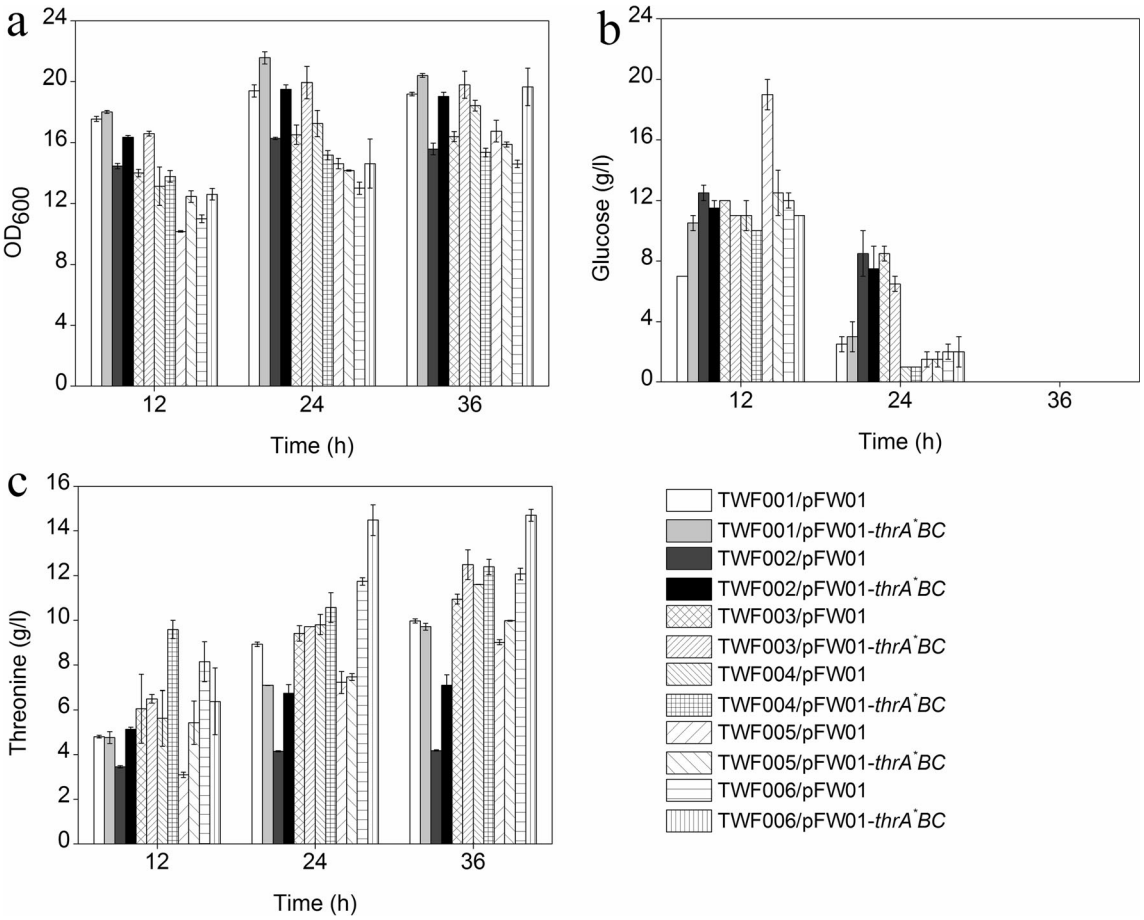

Fig. 4 Flask cultivation for L-threonine production in different *E. coli* strains TWF001/pFW01, TWF001/pFW01-*thrA***BC*, TWF002/pFW01, TWF002/pFW01-*thrA***BC*, TWF003/pFW01, TWF003/pFW01-*thrA***BC*, TWF004/pFW01, TWF004/pFW01-*thrA***BC*, TWF005/pFW01, TWF005/pFW01-*thrA***BC*, TWF006/pFW01, and TWF006/pFW01-*thrA***BC*. **a** Cell growth. **b** Glucose consumption. **c** L-threonine production. The error bars indicate the standard deviations from three independent experiments

*thrA***BC*, TWF006/pFW01, and TWF006/pFW01-*thrA***BC*. The production of L-threonine in these strains was investigated (Fig. 4).

Compared to the vector control TWF001/pFW01, TWF001/pFW01-*thrA***BC* grew better, consumed less glucose, but L-threonine production (9.72 g/l after 36 h) was not increased (Fig. 4). Compared to the vector control TWF002/pFW01, TWF002/pFW01-*thrA***BC* grew better, consumed more glucose, and L-threonine production was higher (Fig. 4). Compared to the vector control TWF003/pFW01, TWF003/pFW01-*thrA***BC* grew better, consumed more glucose, and L-threonine production (12.5 g/l after 36 h) was increased (Fig. 4). Compared to the vector control TWF004/pFW01, TWF004/pFW01-*thrA***BC* grew worse, consumed more glucose, and L-threonine production (12.4 g/l after 36 h) was increased (Fig. 4). Compared to the vector control TWF005/pFW01, TWF005/pFW01-*thrA***BC* grew worse, consumed more glucose, and L-threonine production was higher (Fig. 4). Compared to the vector control TWF006/pFW01, TWF006/pFW01-*thrA***BC* grew better, consumed more glucose, and L-threonine production (14.7 g/l after 36 h) was increased (Fig. 4). Since TWF006/pFW01-*thrA***BC* produced the highest level of L-threonine among all the six strains, it was further investigated.

Comparison of L-threonine production in different *E. coli* TWF006 derivatives

To further increase L-threonine production, the gene *asd* encoding aspartate semialdehyde dehydrogenase and three genes encoding the L-threonine exporters (Yuzbashev et al. 2013) were overexpressed in TWF006, together with the genes *thrA***BC*, resulting in pFW01-*thrA***BC-asd*, TWF006/pFW01-*thrA***BC-rhtA*, TWF006/pFW01-*thrA***BC-rhtC*, and TWF006/pFW01-*thrA***BC-thrE*. RhtA and RhtC were two of the most efficient threonine exporters in *E. coli* (Diesveld et al. 2009; Livshits et al. 2003), while ThrE encoded by *C. glutamicum thrE* can efficiently export L-threonine in *E. coli* (Kruse et al. 2002).

Compared to the control TWF006/pFW01, the growth of pFW01-*thrA***BC-asd*, TWF006/pFW01-*thrA***BC-rhtA*, TWF006/pFW01-*thrA***BC-rhtC*, and TWF006/pFW01-*thrA***BC-thrE* in the medium containing 30 g/l glucose was improved, and glucose was completely consumed in 36 h. After 36 h, L-threonine production in TWF006/pFW01-*thrA***BC-asd*, TWF006/pFW01-*thrA***BC-rhtA*, TWF006/pFW01-*thrA***BC-rhtC*, and TWF006/pFW01-*thrA***BC-thrE* reached 15.85, 14.14, 14.34, and 14.11 g/l, respectively (Fig. 5c). The highest yield of L-threonine was produced by TWF006/pFW01-*thrA***BC-asd*, which is a 70% increase compared to the control TWF001.

Discussion

Engineering the glyoxylate bypass, one of the important anaplerotic pathways, has been used to increase the production of various products. The *iclR* was knocked out in *E. coli* to overcome acetate overflow and improve the production of two acetyl-CoA-derived chemicals, phloroglucinol and 3-hydroxypropionate; the acetate concentrations were decreased by more than 50%, and the productions of the two chemicals were increased more than twice (Liu et al. 2017). Enhancing the glyoxylate shunt by deleting *iclR* has been used in *E. coli* to produce succinate from acetate as the sole carbon source (Li et al. 2016) and to accumulate oxaloacetate precursor to produce L-threonine (Lee et al. 2007), ectoine (Ning et al. 2016), fumaric acid (Zhang et al. 2015b; Song et al. 2013), or fatty acids (Lin et al. 2013). In addition, enhancing the glyoxylate bypass can also be achieved by replacing the native promoter of *aceBAK* with the *trc* promoter in the chromosome (Li et al. 2013), overexpressing the *aceBA* in plasmid (Li et al. 2014), or reducing the enzyme activity of IDH (Li et al. 2013). In this study, either deleting *iclR* or replacing the native promoter of *aceBAK* with the *trc* promoter was performed in L-threonine producing *E. coli* TWF001; L-threonine production was increased in the former case, but decreased significantly in the latter (Fig. 3). This indicates that the engineering of glyoxylate bypass could have different influence in different strains, and the L-threonine production in *E. coli* TWF001 might be regulated by *iclR* as well as other regulators such as *crp* and *arcA* (Yamamoto and Ishihama 2003; Zhang et al. 2005).

Aspartate aminotransferase encoded by *aspC* catalyzes the reaction from oxaloacetate to L-aspartate, drawing the carbon flux from Krebs cycle to the biosynthetic pathway of L-aspartate family. Deletion of *aspC* led to generation of small cells with slow growth, while overexpression of *aspC* exerted the opposite effect (Liu et al. 2014). Therefore, *aspC* was overexpressed in *E. coli* mutant strains TWF003 and TWF004, resulting in TWF005 and TWF006, respectively (Fig. 2). Interestingly, L-threonine production was increased in TWF006, but decreased in TWF005 (Fig. 3). It suggests that enhancing both the glyoxylate bypass and the aspartate aminotransferase could increase the production of threonine in *E. coli*.

Overexpressing the *thrABC* operon is the most often used approach to increase L-threonine production. Since aspartate kinase I encoded by *thrA* can be feedback inhibited, various mutants of *thrA* which can remove the feedback inhibition have been discovered (Dong et al. 2011; Lee et al. 2003; Livshits et al. 2003). The plasmid pFW01-*thrA***BC* (*thrA**is a feedback-resistant *thrA* mutant in which the 1034th C is replaced with T) was transformed into *E. coli* strains TWF001, TWF002, TWF003, TWF004, TWF005, and TWF006. Compared to the vector control, the production of threonine increased in TWF002/pFW01-*thrA***BC*,

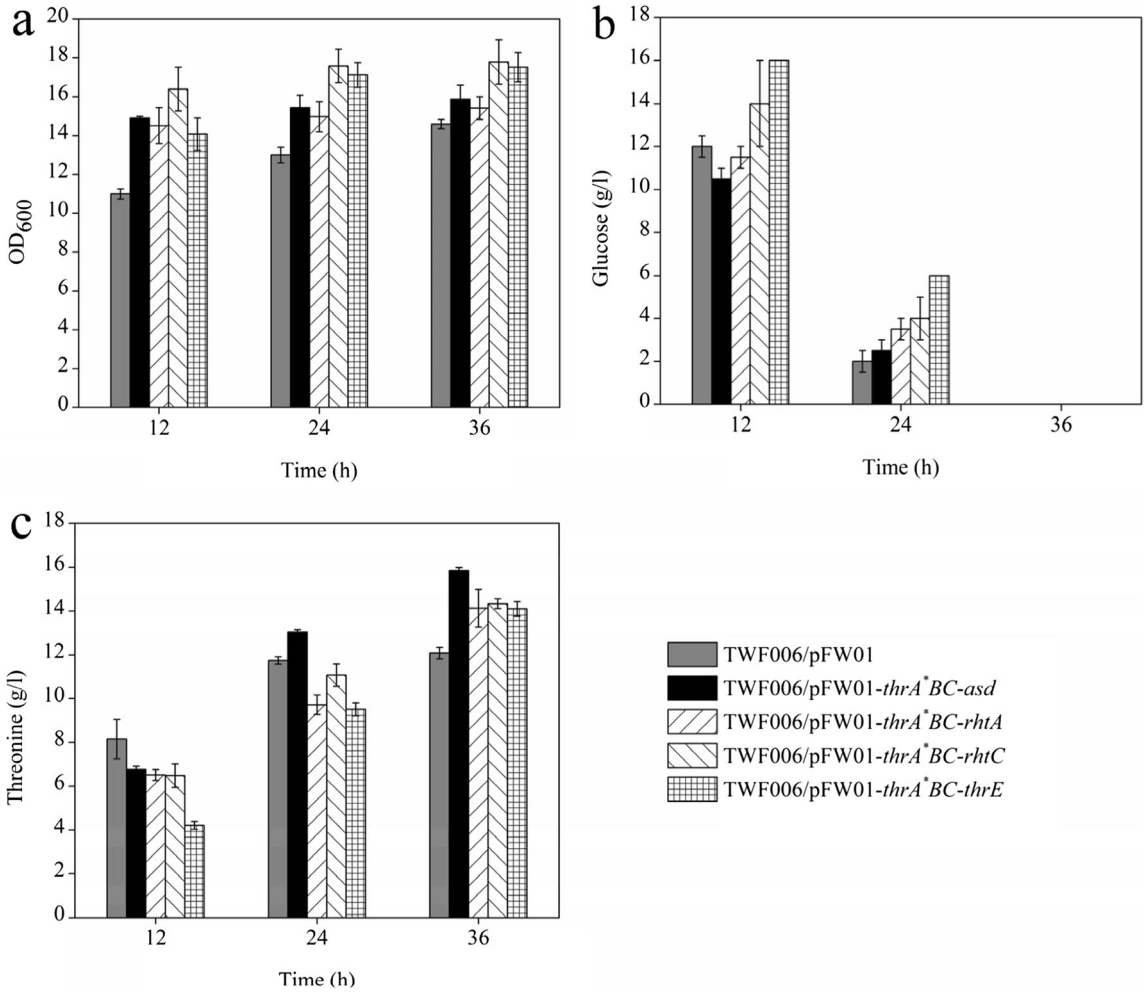

Fig. 5 Flask cultivation for L-threonine production in different *E. coli* strains TWF006/pFW01, TWF006/pFW01-*thrA***BC-asd*, TWF006/pFW01-*thrA***BC-rhtA*, TWF006/pFW01-*thrA***BC-rhtC*, and TWF006/pFW01-*thrA***BC-thrE*. **a** Cell growth. **b** Glucose consumption. **c** L-threonine production. The error bars indicate the standard deviations from three independent experiments

TWF003/pFW01-*thrA***BC*, TWF004/pFW01-*thrA***BC*, TWF005/pFW01-*thrA***BC*, or TWF006/pFW01-*thrA***BC*; while the production of threonine did not increase in the control TWF001/pFW01-*thrA***BC*. This indicates that the various activation of glyoxylate bypass in *E. coli* TWF001 could increase the carbon flow to the L-threonine biosynthetic pathway.

Various strategies have been used to increase L-threonine production in *E. coli*. Based on transcriptome profiling and in silico flux response analysis, a genetically defined L-threonine overproducing *E. coli* strain has been constructed by using systems metabolic engineering (Lee et al. 2007). Firstly, feedback inhibitions of aspartate kinase I and III and transcriptional attenuation regulations were removed; secondly, pathways for Thr degradation were removed by deleting *tdh* and mutating *ilvA*; thirdly, *metA* and *lysA* were deleted to make more precursors available for Thr biosynthesis; finally, the expression levels of some target genes identified by transcriptome profiling combined with in silico flux response analysis were manipulated. The final strain was able to produce 0.393 g L-threonine from 1 g glucose after 50-h fed-batch fermentation (Lee et al. 2007). Another L-threonine hyper-producer MDS-205 was constructed from a reduced-genome *E. coli* strain MDS42, by deleting the gene *tdh*, replacing the genes *sstT* and *tddC* with the gene *rhtA*23, and overexpressing the feedback-resistant threonine operon *thrA***BC*, it could produce 0.401 g L-threonine from 1 g glucose after 30-h fermentation (Lee et al. 2009). The by-product acetate could be reduced in *E. coli* THRD by deleting the genes *pykF* and *pykA* to redirect the glycolysis flux to the pentose phosphate pathway, but the L-threonine yield on glucose (0.34 g/g) did not increase (Xie et al. 2014). Label-free proteomics technology combined with absolute protein expression measurements indicate that LysC has the largest flux control coefficient on L-threonine synthesis in strain *Thr*; therefore, overexpressing LysC can be used to increase L-threonine production (Zhang et al. 2015a). DNA scaffold system also can be used to improve L-threonine production in *E. coli* (Lee et al. 2013). A zinc

finger protein was used as an adapter for the site-specific binding of enzymes involved in L-threonine production to a precisely ordered location on a DNA double helix, thus increasing the proximity of enzymes and the local concentration of metabolites in the cell. Such a DNA scaffold system enhanced the cell growth rate, significantly increased the efficiency of the threonine biosynthetic pathway in *E. coli*, substantially reducing the production time for L-threonine by over 50% (Lee et al. 2013). The fed-batch fermentation technique could also improve the yield of L-threonine in *E. coli*; under the optimal conditions, 0.46 g L-threonine could be obtained from 1 g glucose in *E. coli* TRFC after 38-h fermentation (Chen et al. 2009); combining the pseudo-exponential feeding and glucose-stat feeding strategies resulted in high cell density and more L-threonine production as well as low accumulation of by-products in *E. coli* TRFC (Wang et al. 2014). In this study, one of the strains we constructed, TWF006/pFW001-*thrA*BC-asd*, could produce 0.53 g L-threonine from 1 g glucose in 36-h fermentation. This suggests that enhancing the glyoxylate bypass followed by strengthening the L-threonine biosynthetic pathway is alternative strategy for increasing L-threonine production in *E. coli.* Since overexpression of genes encoding the pyruvate dehydrogenase complex (Skorokhodova et al. 2013), AspA (Chao et al. 2000; Leuchtenberger et al. 2005; Song et al. 2015), or LysC (Zhang et al. 2015a) could increase L-threonine production in *E. coli*, these genes should be overexpressed in TWF006/pFW001-*thrA*BC-asd* in further study to improve L-threonine production, together with fermentation optimization.

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Compliance with ethical standards

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

Compliance with ethics requirements This article does not contain any studies with human participants or animals performed by any of the authors.

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