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Attenuating L-lysine production by deletion of *ddh* and *lysE* and their effect on L-threonine and L-isoleucine production in *Corynebacterium glutamicum*

Xunyan Dong[a,b], Yue Zhao[a,b], Jinyu Hu[a,b], Ye Li[a,b], Xiaoyuan Wang[a,b,c,*]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, China
[b] Key Laboratory of Industrial Biotechnology of Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China
[c] Synergetic Innovation Center of Food Safety and Nutrition, Jiangnan University, Wuxi, China

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ABSTRACT

The fermentative production of L-threonine and L-isoleucine with *Corynebacterium glutamicum* is usually accompanied by the by-production of L-lysine, which shares partial biosynthesis pathway with L-threonine and L-isoleucine. Since the direct precursor for L-lysine synthesis, diaminopimelate, is a component of peptidoglycan and thus essential for cell wall synthesis, reducing L-lysine by-production could be troublesome. Here, a basal strain with eliminated L-lysine production was constructed from the wild type *C. glutamicum* ATCC13869 by deleting the chromosomal *ddh* and *lysE*. Furthermore, the basal strain as well as the *ddh* single mutant strain was engineered for L-threonine production by over-expressing *lysC1*, *hom1* and *thrB*, and for L-isoleucine production by over-expressing *lysC1*, *hom1*, *thrB* and *ilvA1*. Fermentation experiments with the engineered strains showed that (i) deletion of *ddh* improved L-threonine production by 17%, and additional deletion of *lysE* further improved L-threonine production by 28%; (ii) deletion of *ddh* improved L-isoleucine production by 8% and improved cell growth by 21%, whereas additional deletion of *lysE* had no further influence on both L-isoleucine production and cell growth; (iii) L-lysine by-production was reduced by 95% and 86% in L-threonine and L-isoleucine production, respectively, by deletion of *ddh* and *lysE*. This is the first report on improving L-threonine and L-isoleucine production by deleting *ddh* and *lysE* in *C. glutamicum*. The results demonstrate deletion of *ddh* and *lysE* as an effective strategy to reduce L-lysine by-production without surrendering the cell growth of *C. glutamicum*.

1. Introduction

Corynebacterium glutamicum is the leading bacterium for microbial production of amino acids in industry [1,2]. It is non-pathogenic, generally regarded as safe (GRAS), and thus especially suitable for production of pharmaceutical-grade bio-chemicals. The discovery of this gram-positive soil bacterium as a natural hyper-producer of L-glutamate [3] has stimulated great interests in microbial production of both natural and non-natural bio-chemicals and continuing efforts in production strain development [4–6]. Currently, the two most demanding L-amino acids, L-glutamate and L-lysine, are exclusively produced by *C. glutamicum* [7]. Furthermore, *C. glutamicum* strains are developed for the production of L-arginine [8], L-valine [9,10], L-isoleucine [11], L-threonine [12], etc. L-threonine and L-isoleucine are both essential amino acids for mammals that have wide applications in industry. L-threonine is mostly used as a feed additive, as it is the second or third limiting amino acid in animal feeds [12]. Moreover, L-threonine is used as a precursor for synthesis of high value-added drug intermediates [13]. L-isoleucine is a branched-chain amino acid (BCAA) mostly used as a constituent of infusions in pharmaceutical industry [14,15]. The high value-added L-isoleucine is also expected for feed-use in the future, as long as the production cost is reduced and the market price drops [14].

L-threonine and L-isoleucine both belong to the aspartic family of amino acids, and L-threonine serves as a precursor for L-isoleucine biosynthesis. Starting from L-aspartate, the biosynthesis of L-threonine involves five enzymatic steps (Fig. 1). In *C. glutamicum*, L-threonine biosynthesis is controlled by the three key enzymes aspartate kinase (AK, encoded by *lysC*), homoserine dehydrogenase (HD, encoded by *hom*) and homoserine kinase (HK,

* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, 1800 Lihu Avenue, Wuxi 214122, China.
E-mail address: xiaoyuanwang@hotmail.com (X. Wang).

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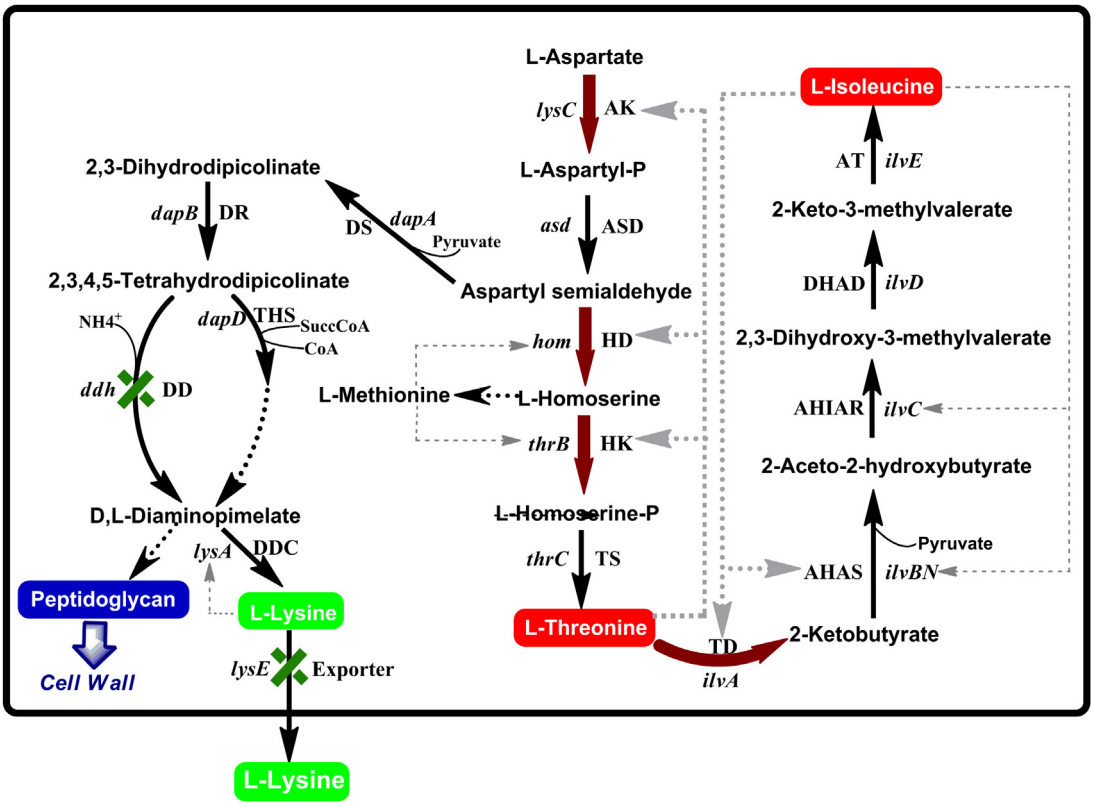

Fig. 1. The biosynthesis pathway of L-threonine and L-isoleucine in *C. glutamicum*. Black dashed arrows indicate more than one enzymatic step; bold grey dashed arrows indicate feed-back inhibition; plain grey dashed arrows indicate feed-back repression; dark red arrows indicate that the corresponding genes were over-expressed; dark green Xs indicate that the corresponding genes were knocked out. AK, aspartate kinase; ASD, aspartyl semialdehyde dehydrogenase; HD, homoserine dehydrogenase; HK, homoserine kinase; TS, threonine synthase; DS, dihydrodipicolinate synthase; DR, dihydrodipicolinate reductase; THS, tetrahydrodipicolinate succinylase; DD, diaminopimelate dehydrogenase; DDC, diaminopimelate decarboxylase; LysE, L-lysine exporter; TD, threonine dehydratase; AHAS, acetohydroxy acid synthase; AHIAR, acetohydroxy acid isomeroreductase; DHAD, dihydroxy acid dehydratase; AT, branched chain acid aminotransferase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

encoded by *thrB*), respectively [12]. Starting from L-threonine, the biosynthesis of L-isoleucine involves another five enzymatic steps (Fig. 1). In *C. glutamicum*, L-isoleucine biosynthesis is controlled by threonine dehydratase (TD, encoded by *ilvA*) and acetohydroxy acid synthase (AHAS, encoded by *ilvBN*) in addition to AK, HD and HK [16]. L-lysine and L-methionine branches compete with L-threonine and L-isoleucine pathways for carbon flux at the nodes of L-aspartyl semialdehyde and L-homoserine, respectively (Fig. 1). The L-lysine branch is initiated by dihydrodipicolinate synthase (DS, encoded by *dapA*), and splits into two parallel variants at tetrahydrodipicolinate: the diaminopimelate dehydrogenase (DD, encoded by *ddh*) variant and the succinylase variant initiated by tetrahydrodipicolinate succinylase (THS, encoded by *dapD*) [17].

Reducing by-products accumulation is an effective approach to reduce the production cost, as the downstream purification and recovery process could be simplified [18]. Furthermore, reducing certain by-products formation may improve the titer and/or yield of the target product, as the carbon source could be utilized more concentrated for the target product biosynthesis [9]. In L-threonine and L-isoleucine production with *C. glutamicum*, L-lysine is the common main by-product [19,20]. The easy accumulation of L-lysine is due to that (i) the three amino acids share partial biosynthesis pathway (Fig. 1); (ii) unlike the L-threonine and L-isoleucine terminal pathways which are strictly feed-back regulated (Fig. 1), the L-lysine terminal pathway is almost free from feed-back regulation in *C. glutamicum* [21]; (iii) the L-lysine efflux system mainly fulfilled by the specific exporter LysE (encoded by *lysE*) is highly inducible and active [22]. Eliminating L-lysine by-production is very difficult to tackle, since the direct precursor for L-lysine synthesis, D,L-diaminopimelate, is a component of peptidoglycan and thus is essential for cell wall synthesis [23]. The only study concerning reducing L-lysine by-production in L-isoleucine production with *C. glutamicum* was recently reported by Vogt et al. [19], in which the transcription of *dapA* was down-regulated, as a result, the cell growth was substantially retarded and the final cell density was reduced as well.

Maintaining cell robustness is of great significance to industrial production. Thus in this work, we aimed to find another solution to reduce L-lysine accumulation in L-threonine and L-isoleucine production with *C. glutamicum*, without affecting the cell growth.

2. Materials and methods

2.1. Bacteria and culture conditions

All bacteria and plasmids used in this work are listed in Table 1. *E. coli* was grown in Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L trypton and 10 g/L NaCl) at 37 °C and 200 rpm. *C. glutamicum* was grown in LBHIS medium (2.5 g/L yeast extract, 5 g/L tryptone, 18.5 g/L brain heart infusion powder, 5 g/L NaCl and 91 g/L sorbitol) [24] at 30 °C and 200 rpm, if not specified elsewhere. For preparation of competent cells, *C. glutamicum* was grown in modified Epo medium (0.1% Tween 80, 30 g/L glycine, 5 g/L yeast extract, 10 g/L trypton and 10 g/L NaCl) [5]. When necessary, 50 μg/mL kanamycin was supplemented in the medium for recombinant *E. coli* to maintain the plasmid; 30 μg/mL kanamycin or 10 μg/mL chloramphenicol was supplemented in the medium for recombinant *C. glutamicum* to maintain plasmids.

Table 1
Bacterial strains and plasmids used in this work.

Strains or plasmids	Description	Sources
Strains		
JM109	Wild type *E. coli*	NEB
ATCC13869	Wild type *C. glutamicum*	ATCC
XDW101	ATCC13869 Δ*ddh*	This work
XDW102	ATCC13869 Δ*ddh* Δ*lysE*	This work
XDW103	ATCC13869 Δ*lysE*	This work
TDW101	ATCC13869 harboring pDXW-8-*lysC1-hom1-thrB1*	[20]
TDW102	XDW101 harboring pDXW-8-*lysC1-hom1-thrB1*	This work
TDW103	XDW102 harboring pDXW-8-*lysC1-hom1-thrB1*	This work
TDW104	XDW103 harboring pDXW-8-*lysC1-hom1-thrB1*	This work
IDW101	ATCC13869 harboring pDXW-8-*lysC1-hom1-thrB1-ilvA1*	[20]
IDW102	XDW101 harboring pDXW-8-*lysC1-hom1-thrB1-ilvA1*	This work
IDW103	XDW102 harboring pDXW-8-*lysC1-hom1-thrB1-ilvA1*	This work
IDW104	XDW103 harboring pDXW-8-*lysC1-hom1-thrB1-ilvA1*	This work
Plasmids		
pDXW-8-*lysC1-hom1-thrB1*	Shuttle expression plasmid pDXW-8 with *lysC1* and *hom1-thrB1* operon of *C. glutamicum* IWJ001; Km^r	[20]
pDXW-8-*lysC1-hom1-thrB1-ilvA1*	pDXW-8 with *lysC1*, *hom1-thrB1* operon, and *ilvA1* of *C. glutamicum* IWJ001; Km^r	[20]
pBluescriptIISK(+)	Cloning vector, ColE, lacZ, Amp^r	NEB
pDTW109	Temperature sensitive Cre expression vector	[26]
pDTW201	pBluescriptIISK(+) with *loxL-kan-loxR* segment	[26]
pDW1d	*ddh* deletion vector derived from pBluescriptIISK(+), Amp^r, Km^r	This work
pDW2d	*lysE* deletion vector derived from pBluescriptIISK(+), Amp^r, Km^r	This work

Amp^r:Ampicillinresistance;Km^r:Kanamycinresistance.

2.2. Construction of plasmids

Plasmids pDW1d and pDW2d (Fig. 2) were constructed for deletion of *ddh* and *lysE*, respectively, in the chromosome of *C. glutamicum*. Primers used in this work are listed in Table 2. To construct pDW1d, the flanking upstream (1004 bp) and downstream (1036 bp) fragments of *ddh* were amplified from the genomic DNA of *C. glutamicum* ATCC13869 with primer pairs Δ*ddh*-U-F/Δ*ddh*-U-R and Δ*ddh*-D-F/Δ*ddh*-D-*R*, respectively; the *loxL-kan-loxR* fragment was amplified from pDTW201 with primer pair *kan-lox*-F/*kan-lox*-R; then the three fragments were separately digested with appropriate enzymes, ligated together, and cloned into the *Sal*I and *Pst*I sties of pBluescriptIISK(+). To construct pDW2d, the flanking upstream (1008 bp) and downstream (999 bp) fragments of *lysE* were amplified from the genomic DNA of *C. glutamicum* ATCC13869 with primer pairs Δ*lysE*-U-F/Δ*lysE*-U-R and Δ*lysE*-D-F/Δ*lysE*-D-*R*, respectively; then constructed similarly as pDW1d.

Primer synthesis was performed by Sangon (Shanghai, China). TIANamp bacteria DNA kit for genomic DNA isolation was purchased from Tiangen (Beijing, China). PrimeSTAR HS DNA polymerase for PCR amplification was purchased from Takara (Dalian, China). Restrictions enzymes and T4 DNA ligase were purchased from Sangon (Shanghai, China).

Table 2
Primers used in this work.

Primers	Nucleotide sequences (5′→3′)	Restriction sites
kan-lox-F	ATGGATCCAATACGACTCACTATAGG	*BamH*I
kan-lox-R	ACCTCTAGAGCGCAATTAACCCTCACTAAAG	*Xba*I
Δ*ddh*-U-F	ATTAGTCGACAATCGCTCAAGGCTGCTGCTGACCT	*Sal*I
Δ*ddh*-U-R	ATTAGGATCCAGGTTTCCGTAGCCCACGATAGCTA	*BamH*I
Δ*ddh*-D-F	ATTATCTAGAAAGTTGCTCCATACCTGCTCTCCCC	*Xba*I
Δ*ddh*-D-R	ATTACTGCAGCAAACCCATCTTTAGCCAGATCCACC	*Pst*I
Δ*ddh*-V-F	ATCATTCCAATGCTGATCCACCCCA	
Δ*ddh*-V-R	ACATGGGTATCGGCCTAGGTTGGT	
Δ*lysE*-U-F	ATTAGTCGACAGAGATCTAGATTCCAGGCGCC	*Sal*I
Δ*lysE*-U-R	ATTAGGATCCGAGAAGAACCGCAATGAGTCC	*BamH*I
Δ *lysE*-D-F	ATTCTCTAGAAGAGATCTAGATTCCAGGCGCC	*Xba*I
Δ *lysE*-D-R	ATGCCTGCAGGAGAAGAACCGCAATGAGTCC	*Pst*I
Δ *lysE*-V-F	CAGCAAGGCGTCCAGATAGTTG	
Δ *lysE*-V-R	TGGACAACAGCCTTGATTCACTC	
16S rRNA-F	ACCTGGAGAAGAAGCACCG	
16S rRNA-R	TCAAGTTATGCCCGTATCG	
ddh-F	TGCTGTTCCTGTGCATGGGC	
ddh-R	GCGGTGGTTGTCGTAGGTGT	
lysE-F	GTGGCATCGCTTACCTGTTATG	
lysE-R	GCACGGTTGGTTCTGTTTCTTCA	
ilvA-F	GCAGGCACGAATTTCCTCC	
ilvA-R	CGGATCTTGTAGGAACGAAC	
lysC-F	GATATGCTCCTGACTGCTGG	
lysC-R	CAACAATGCGTGCGTTTCC	
hom-F	GTGATGAACTTGCGCACCG	
hom-R	GAGTGCAAAAGCGTCCTCAG	
thrB-F	TATTCGTGCTGGCCTGAGG	
thrB-R	GCCAAACCATTAGCTGCAGC	

The restriction sites are underlined.

2.3. Construction of strains

For construction of *C. glutamicum* strains, preparation of competent cells and electroporation were performed by the published method [25]. XDW101 was constructed from *C. glutamicum* ATCC13869 by deleting the chromosomal *ddh*. Deletion of *ddh* was realized by using the Cre-*loxP* system [26] (Fig. 2). Firstly, *C. glutamicum* ATCC13869 was transformed with the plasmid pDW1d by electroporation and selected for kanamycin resistance on LBHIS plate supplemented with 30 μg/mL kanamycin. Colonies were picked and verified by PCR with primer pair Δ*ddh*-V-F/Δ*ddh*-V-R to confirm the correct insertion of *loxL-kan-loxR* in the chromosome. Secondly, the correct recombinant strain was transformed with the temperature-sensitive plasmid pDTW109 and selected for chloramphenicol resistance on LBHIS plate supplemented with 10 μg/mL chloramphenicol at 25 °C. Colonies were picked and verified by PCR with primer pair Δ*ddh*-V-F/Δ*ddh*-V-R to confirm the removal of *loxL-kan-loxR* from the chromosome. Finally, the correct recombinant colony was inoculated into liquid LBHIS medium and incubated at 37 °C, 200 rpm to induce the simultaneous loss of pDTW109. After 36 h cultivation, the culture was streaked on LBHIS plates and incubated at 30 °C. Colonies were picked and each was streaked on three LBHIS plates: (i) without any antibiotic; (ii)

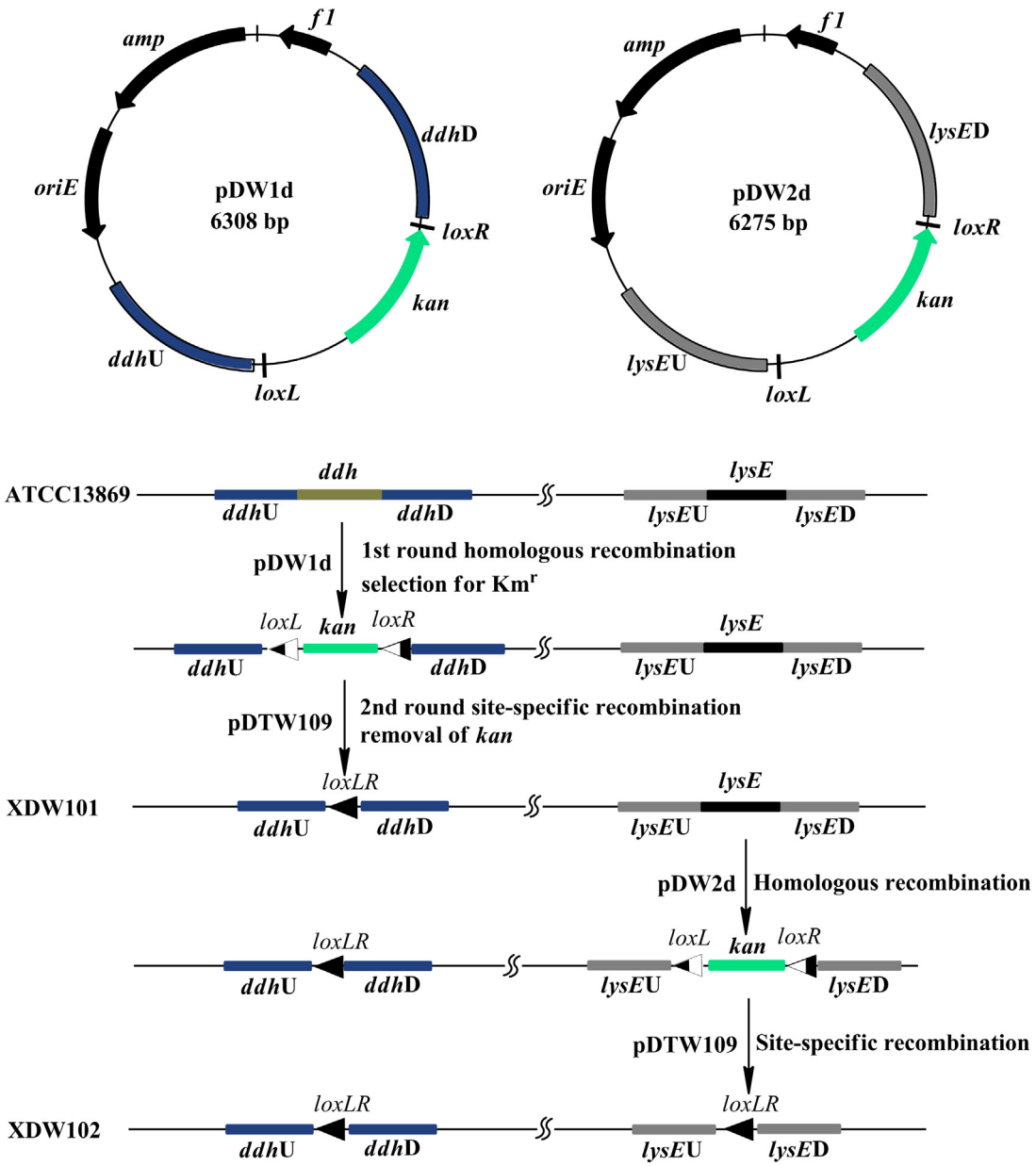

Fig. 2. Maps of deletion vectors pDW1d and pDW2d and the process for deleting the chromosomal *ddh* and *lysE*. *oriE*, the origin of *E. coli* plasmid pBR322; *amp*, ampicillin resistance gene; *kan*, kanamycin resistance gene; *ddh*U, the chromosomal upstream flanking region of *ddh*; *ddh*D, the chromosomal downstream flanking region of *ddh*; *lysE*U, the chromosomal upstream flanking region of *lysE*; *lysE*D, the chromosomal downstream flanking region of *lysE*.

with chloramphenicol; (iii) with kanamycin. The colony with sensitivity to both chloramphenicol and kanamycin was designated as XDW101. Similarly, XDW102 and XDW103 were constructed from XDW101 and ATCC13869 by deleting the chromosomal *lysE*, respectively(Fig. 2).

TDW102, TDW103 and TDW104 were constructed by introducing the recombinant expression plasmid pDXW-8-*lysC1-hom1-thrB1* (Fig. 3) into XDW101, XDW102 and XDW103, respectively. IDW102, IDW103 and IDW104 were constructed by introducing the recombinant expression plasmid pDXW-8-*lysC1-hom1-thrB1-ilvA1* (Fig. 3) into XDW101, XDW102 and XDW103, respectively.

2.4. Fermentation

The seed medium (pH7.0) contained 25 g/L glucose, 1.25 g/L urea, 20 g/L corn steep liquor, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ and 30 μg/mL kanamycin for maintaining the plasmid. The fermentation medium (pH7.0) contained 80 g/L glucose, 35 g/L ammonium sulfate, 20 g/L corn steep liquor, 1 g/L KH_2PO_4, 1 g/L $MgSO_4$, 30 g/L $CaCO_3$ for pH buffering, 30 μg/mL kanamycin for maintaining the plasmid and 1 mM IPTG for induction.

For batch cultivation, each strain was streaked out from a frozen glycerol stock onto an LBHIS plate and incubated at 30 °C for 48 h. A single colony from the LBHIS plate was picked and inoculated into 25 mL seed medium in 250 mL flask. The seed culture was incubated at 30 °C and 180 rpm for 18 h. Then the seed culture was inoculated into 25 ml fermentation medium in 250 mL baffled flask, giving an initial optical density at 562 nm (OD_{562}) of 0.1. The fermentation culture was incubated at 30 °C and 180 rpm for 72 h. Samples were taken every 6 h at the exponential phase and every 12 h at the stationary phase from each culture, for analysis of cell growth, glucose consumption and amino acids production.

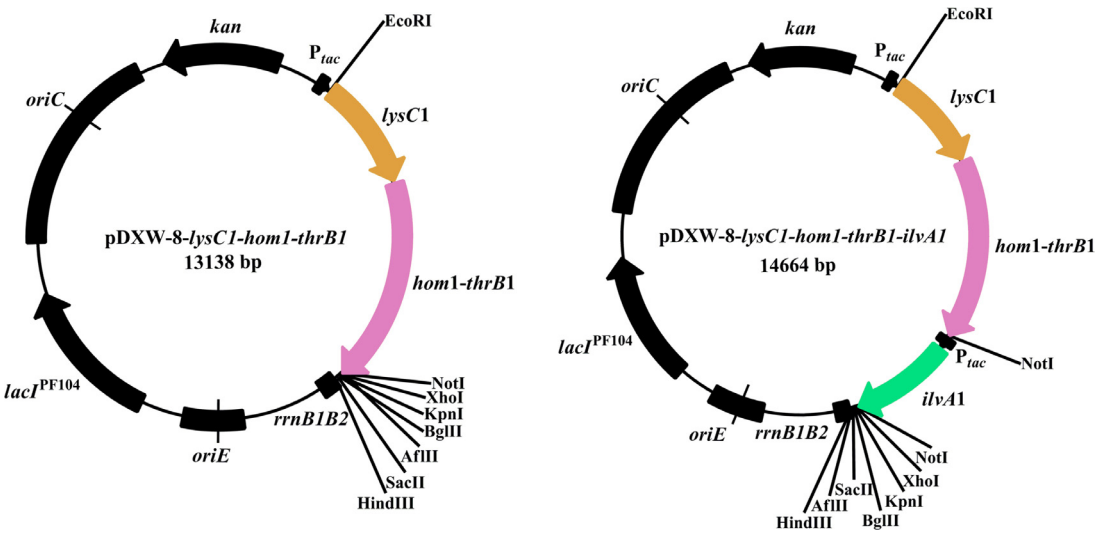

Fig. 3. Maps of expression vectors pDXW-8-*lysC1-hom1-thrB1* and pDXW-8-*lysC1-hom1-thrB1-ilvA1* used in this work. *oriE*, the origin of *E. coli*plasmid pBR322; *oriC*, the origin of *C. glutamicum* plasmid pC2; *kan*, kanamycin resistance gene; P*tac*, *tac* promoter; *rrnBT1T2*, the transcriptional terminator from *E. coli* plasmid pKK223-3.

2.5. Quantification of mRNA using real-time PCR

Real-time reverse transcription PCR (RT-PCR) was performed to analyze relative transcription levels of genes in different strains. Fermentation samples of strains collected at the mid-exponential phase were used for RT-PCR experiments. Extraction of total RNA, synthesis of cDNA and RT-PCR with an ABI Step One RT-PCR system (Applied Biosystems, San Mateo, CA, USA) were performed according to the published method [9]. To normalize the results, the relative abundance of 16S rRNA was taken as the internal standard control. RNA extraction kit was purchased from BioFlux (Beijing, China); DNase I was purchased from TaKaRa (Dalian, China); Revert AidTM First Strand cDNA synthesis kit was purchased from Fermentas (Shanghai, China); Real Master Mix kit was purchased from TIANGEN (Beijing, China).

2.6. Analytical procedures

The cell growth of *C. glutamicum* was monitored by measuring OD_{562} on an UV-1800 spectrophotometer (Shimadzu, Japan). The glucose concentration was measured on an SBA-40 biosensor using the dinitrosalicylic acid method (Key Laboratory of Biosensor of Shandong Province, China). Amino acids concentrations were measured by the HPLC method [27]. Each sample taken from the fermentation broth was centrifuged at 12,000 rpm for 30 min. The supernatant was collected, diluted 25 folds, filtered and then analyzed by HPLC on the 1200 Series HPLC system (Agilent Technology, USA) with a Synergi 4 μm column (MAX-RP 80 Å, 25 × 4.6 mm) (Phenomenex, Aschaffenburg, Germany). All the solvents used in HPLC analysis were purchased from Sigma-Aldrich (USA). For determination of the intracellular L-lysine concentration, the washed precipitate was resuspended in phosphate buffer (20 mM, pH 7.2) and boiled for 15 min, then centrifuged at 12,000 rpm for 10 min to remove cell debris; the supernatant was used for HPLC analysis. The dry cell weight (DCW) was calculated from OD_{562}, according to the experimentally determined equation: DCW (g/L) = 0.35* OD_{562} + 0.24 [9]. The intracellular volume is 1.6 mL per gram DCW [25].

3. Results and discussion

3.1. Rational selection of target genes by analyzing the L-lysine pathway

L-lysine terminal pathway (Fig. 1) starts with DS (encoded by *dapA*) which condenses L-aspartyl semialdehyde and pyruvate into dihydrodipicolinate. Subsequently, dihydrodipicolinate is converted to tetrahydrodipicolinate by dihydrodipicolinate reductase (DR, encoded by *dapB*). At tetrahydrodipicolinate, the pathway splits into two variants to form D,L-diaminopimelate: (i) the one-step dehydrogenase variant that uses ammonium; (ii) the four-step succinylase variant that uses organic nitrogen compounds [23]. Finally, L-lysine is formed from D,L-diaminopimelate by the catalysis of diaminopimelate decarboxylase (DDC, encoded by *lysA*). D,L-diaminopimelate also serves as a precursor for the synthesis of peptidoglycan, an essential component of the cell wall. *C. glutamicum* uses novel split pathway for L-lysine synthesis probably for flexible utilization of nitrogen sources in different environments to ensure reliable provision of D,L-diaminopimelate for cell wall synthesis [23]. Among the enzymes in the L-lysine terminal pathway, the only feed-back regulation found was the repression of DDC synthesis by L-lysine [28]. The efflux of L-lysine is predominantly fulfilled by the lysine-specific exporter LysE [29].

To reduce L-lysine accumulation, the challenge lies in maintaining adequate D,L-diaminopimelate for cell propagation, as *C. glutamicum* was shown unable to take up D,L-diaminopimelate supplemented in the culture medium [28]. The *dapA* gene is certainly considered as the prime target for modification, as its enzyme product DS controls the total carbon influx of the L-lysine terminal pathway. However, DS has a low affinity for L-aspartyl semialdehyde and a low maximal specific activity [21]. It was reported that attenuating the transcription of *dapA* by approximately 80% by promoter mutation effectively reduced L-lysine by-production in an L-isoleucine producing *C. glutamicum*, but substantially retarded cell growth and reduced final cell density by approximately 25% as well [19]. Moreover, it should be mentioned that Vogt et al. [19] adopted two mutant *dapA* promoters with similar transcriptional strength, but only one of the two led to significant reduction in L-lysine by-production. In addition, in our attempts of substituting the *dapA* promoter with weaker mutant ones, correct recombinant transformants were hardly obtained. In sum, attenuating *dapA*

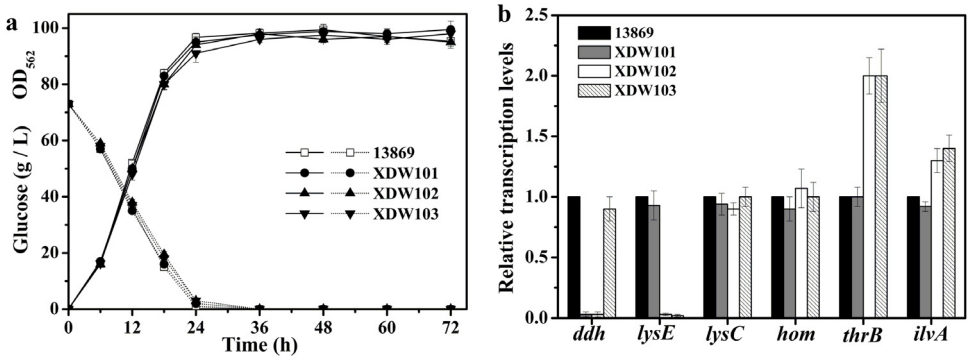

Fig. 4. Effect of *ddh* and *lysE* knockout on the cell growth of *C. glutamicum* ATCC13869. (A) Cell growth (solid lines) and glucose consumption (dotted lines). (B) Relative transcription levels of *ddh*, *lysE*, *lysC*, *hom*, *thrB* and *ilvA* in XDW101, XDW102 and XDW103 investigated by RT-PCR, using 13869 as a control. The shown data represent mean values of three independent experiments with standard deviations.

expression to reduce L-lysine accumulation would be a tailor-made task and requires iterative trials, due to the varied availability of L-aspartyl semialdehyde among strains. Similarly, the *dapB* gene is also positioned upstream of the split and thus was not considered as a target in this work. As for the two split variants, the succinylase variant was found dispensable for both cell growth and L-lysine production [30], whereas the dehydrogenase variant was found influential for L-lysine production, in L-lysine production strains under fermentation conditions [17,31,32]. Thus *ddh* seems to be an attractive target for attenuating L-lysine accumulation. In metabolic engineering of *E. coli* for L-threonine and L-isoleucine production, the strategy of deleting *lysA* encoding the last enzyme in L-lysine biosynthesis was adopted to block L-lysine accumulation [33,34]. As deletion of *lysA* makes the strain auxotrophic for L-lysine, and the expression of *lysA* in *C. glutamium* is already subjected to feed-back repression by L-lysine (Fig. 1), *lysA* was not considered as a target in this work. The LysE mediated export is proven pivotal for L-lysine production in *C. glutamicum* [21,22,35]. Thus *lysE* was chosen as the second target for modification in addition to *ddh*.

3.2. *Effect of* ddh *and* lysE *deletion on cell growth and* L*-lysine production in wild type* C. glutamicum

At the attempt of constructing a basal strain with eliminated L-lysine production for flexible application in metabolic engineering, we chose the wild-type *C. glutamicum* ATCC13869 as the starting strain. The *ddh* gene was first deleted from the chromosome of ATCC13869. The successful deletion of *ddh* was confirmed at the transcription level (Fig. 4). The resulting strain XDW101 exhibited identical cell growth and glucose consumption pattern to the parental ATCC13869 during batch fermentation in flasks (Fig. 4). This result is consistent with the finding reported by Schrumpf et al. [17] that *ddh* is not essential for *C. glutamicum*. After 72 h cultivation, the amino acids concentrations in the fermentation broth were determined. Unexpectedly, XDW101 produced as much

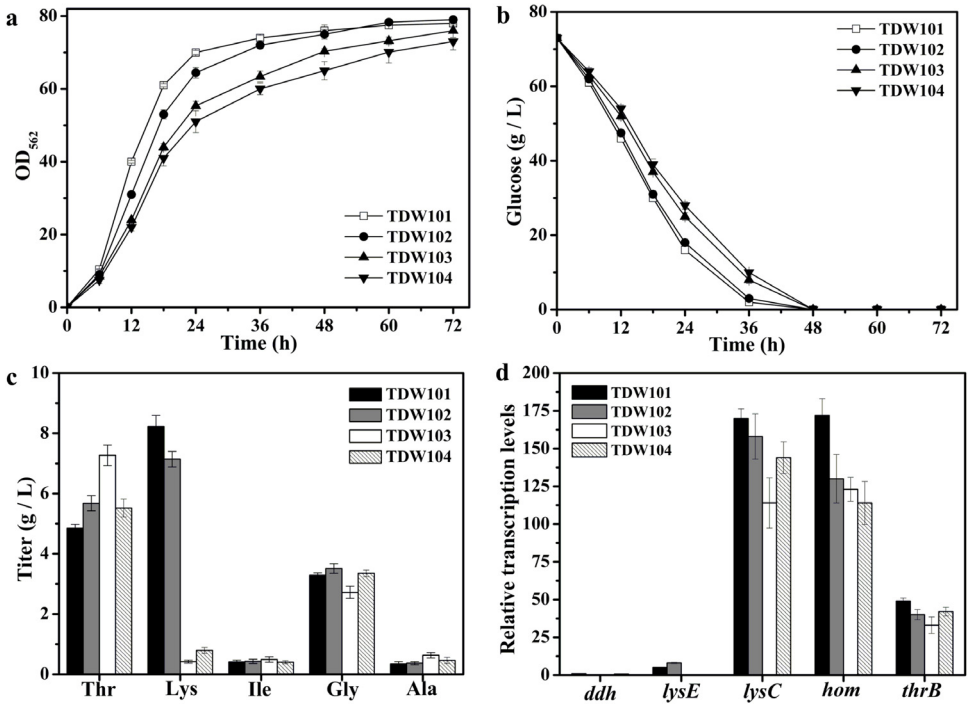

Fig. 5. Effect of *ddh* and *lysE* knockout on L-threonine production in *C. glutamicum* ATCC13869. (A) Cell growth. (B) Glucose consumption. (C) Amino acids production determined after 72 h cultivation. (D) Relative transcription levels of *ddh*, *lysE*, *lysC*, *hom*, and *thrB* in TDW101, TDW102, TDW103 and TDW104 investigated by RT-PCR, using the original 13869 as a control. The shown data represent mean values of three independent experiments with standard deviations.

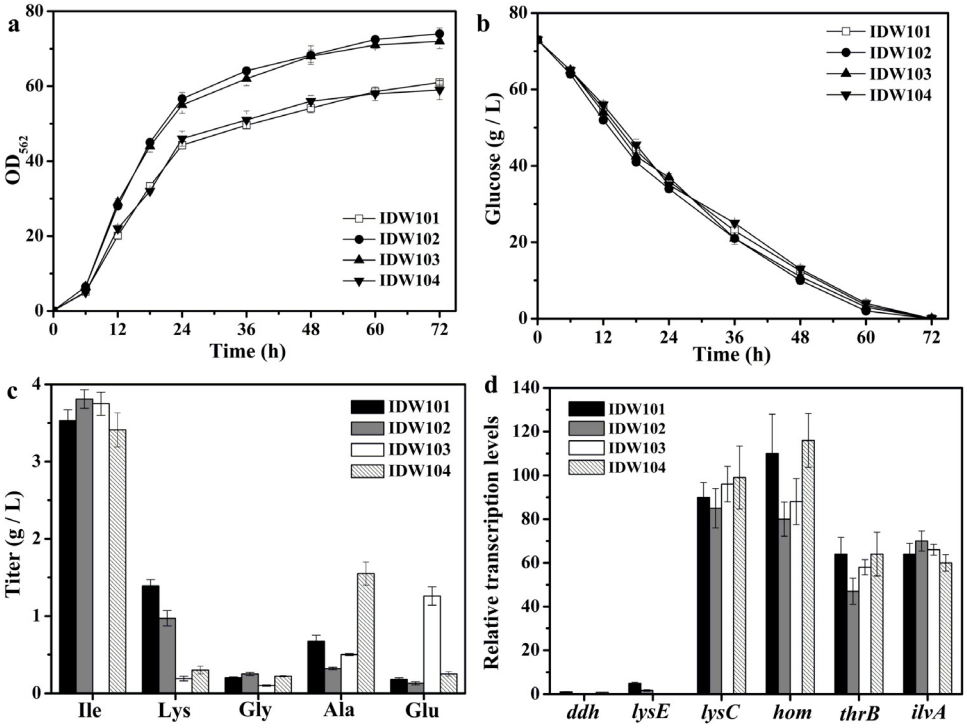

Fig. 6. Effect of *ddh* and *lysE* knockout on L-isoleucine production in *C. glutamicum* ATCC13869. (A) Cell growth. (B) Glucose consumption. (C) Amino acids production determined after 72 h cultivation. (D) Relative transcription levels of *ddh*, *lysE*, *lysC*, *hom*, *thrB* and *ilvA* in IDW101, IDW102, IDW103 and IDW104 investigated by RT-PCR, using the original 13869 as a control. The shown data represent mean values of three independent experiments with standard deviations.

L-lysine as ATCC13869 did (0.36 g/L). In addition, deletion of *ddh* did not alter the products spectrum either. It was reported that the dehydrogenase variant was prerequisite for handing an increased flux flow in the L-lysine pathway [17]. Although the strategy of over-expressing *ddh* was frequently adopted in constructing *C. glutamicum* L-lysine production strains [17,31,32], Shaw-Reid et al. [30] failed to increase L-lysine production in the L-lysine producing ATCC21253 by over-expressing *ddh*. Thus the failure of attenuating L-lysine production by *ddh* deletion here could possibly be ascribed to the low flux in the L-lysine pathway in ATCC13869.

Subsequently, the *lysE* gene was deleted from the chromosome of XDW101. The successful deletion of *lysE* was confirmed at the transcription level (Fig. 4). The resulting strain XDW102 exhibited almost identical cell growth and glucose consumption pattern to the parental XDW101 during batch cultivation (Fig. 4). That is, XDW102 maintained the robustness of the original wild-type ATCC13869. After 72 h cultivation, L-lysine was not detected in the fermentation broth of XDW102. We also constructed the *lysE* single mutant strain XDW103 from ATCC13869 as a reference strain. XDW103 did not excrete L-lysine in the medium during fermentation either.

3.3. *Effect of* ddh *and* lysE *deletion on cell growth and* L-*threonine production in* L-*threonine producing* C. glutamicum

Strains XDW101, XDW102 and XDW103 were then engineered into L-threonine producers to investigate the respective effect of *ddh* deletion and *lysE* deletion on L-threonine production. L-threonine biosynthesis in *C. glutamicum* is mainly controlled by AK (encoded by *lysC*), HD (encoded by *hom*) and HK (encoded by

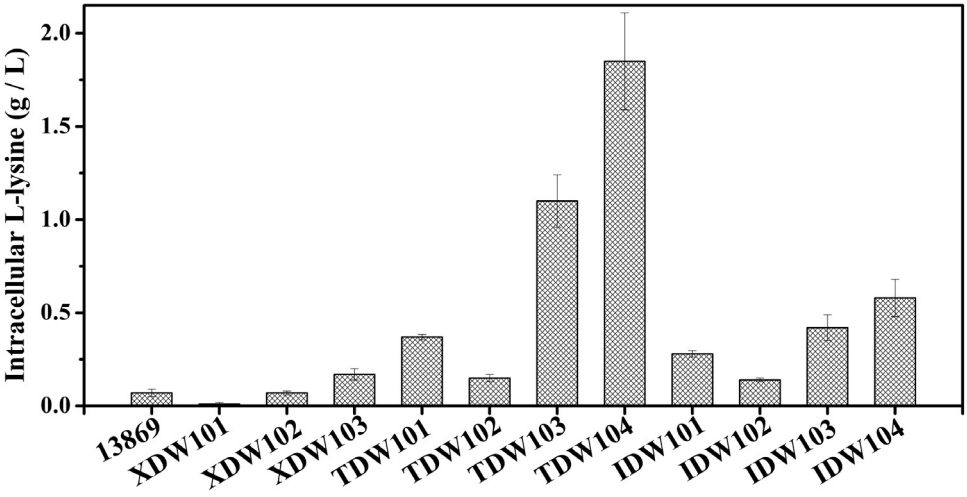

Fig. 7. Intracellular L-lysine concentrations of strains used in this work determined at the end of fermentation.

thrB) (Fig. 1). The three enzymes are all subjected to feed-back inhibition by L-threonine. Releasing the key enzymes from feed-back regulation is proven as the priority to achieve high production of L-threonine [12,33]. Therefore, *lysC1* encoding a feed-back resistant AK mutant, *hom1* encoding a feed-back resistant HD mutant [20] and *thrB* were sequentially over-expressed in XDW101, XDW102 and XDW103, resulting in TDW102, TDW103 and TDW104, respectively. TDW101 derived from ATCC13869 by over-expressing the same three genes was taken as control strain. The successful expression of the three genes was confirmed at the transcription level (Fig. 5D). During batch fermentation, TDW102 exhibited slightly slowed cell growth in comparison to TDW101, but reached the same cell density as TDW101 at the end of cultivation (Fig. 5A). The glucose consumption pattern of TDW102 was identical to that of TDW101 (Fig. 5B). After 72 h cultivation, amino acids concentrations in the fermentation broth were determined (Fig. 5C). The L-threonine production of TDW102 (5.68 g/L) was 17% higher, whereas the L-lysine production (7.14 g/L) was 13% lower than those of TDW101. No obvious change in other by-products (glycine, L-alanine and L-isoleucine) accumulation was observed between TDW102 and TDW101. These results suggested that (i) deletion of *ddh* could only weakly attenuate L-lysine production in this case; (ii) attenuating L-lysine biosynthesis could force the carbon flux into L-threonine biosynthesis and thus enhance L-threonine titer.

TDW103 exhibited slowed cell growth and glucose consumption in comparison to TDW102 and TDW101 (Fig. 5A, B), but reached comparable cell density to TDW102 and TDW101 at the end of fermentation. As for amino acids production, the L-threonine production of TDW103 (7.27 g/L) was 28% and 50% higher than that of TDW102 and TDW101, respectively (Fig. 5C). Whereas the L-lysine production (0.42 g/L) of TDW102 was 94% and 95% lower than that of TDW102 and TDW101, respectively (Fig. 5C). Furthermore, decreased glycine accumulation and increased L-alanine accumulation in TDW103 were observed, as compared with TDW102 and TDW101. In contrast, the L-threonine production of TDW104 (5.52 g/L) was only 14% higher than that of TDW101, indicating combinatorial deletion of *lysE* with *ddh* achieved synergistic effect on enhancing L-threonine production. These results suggested that (i) deletion of *lysE* could substantially reduce but is insufficient to completely eliminate L-lysine production when the pathway flux is high; (ii) deletion of *lysE* could be an effective strategy to improve L-threonine production in *C. glutamicum*; (iii) combinatorial deletion of *lysE* with *ddh* could be an effective strategy to improve the performance of the L-threonine producing *C. glutamicum* by reducing L-lysine accumulation meanwhile maintaining the robustness of the strain.

3.4. *Effect of* ddh *and* lysE *deletion on cell growth and* L-*isoleucine production in* L-*isoleucine producing* C. glutamicum

Strains XDW101, XDW102 and XDW103 were also engineered into L-isoleucine producers to evaluate their suitability for application in L-isoleucine production as a host, and to investigate the respective effect of *ddh* deletion and *lysE* deletion on L-isoleucine production. L-isoleucine biosynthesis in *C. glutamicum* is controlled by five key enzymes that are subjected to feed-back regulation [16]: AK (encoded by *lysC*), HD (encoded by *hom*), HK (encoded by *thrB*), TD (encoded by *ilvA*) and AHAS (encoded by *ilvBN*) (Fig. 1). However, according to previous studies, over-expression of *ilvBN* (either the wild-type or a more feed-back resistant allele) had no contribution to increasing L-isoleucine production in the wild-type *C. glutamicum* [19,20]. Thus *lysC1*, *hom1*, *thrB* and *ilvA1* encoding a feed-back resistant TD mutant were sequentially over-expressed in XDW101, XDW102 and XDW103, resulting in IDW102, IDW103 and IDW104, respectively. IDW101 derived from ATCC13869 by over-expressing the same four genes was taken as control strain. The successful expression of the four genes was confirmed at the transcription level (Fig. 6D). During batch fermentation, IDW102 exhibited accelerated cell growth and reached approximately 21% higher cell density in comparison to IDW101 (Fig. 6A). The glucose consumption pattern of IDW102 was identical to that of IDW101 (Fig. 6B). After 72 h cultivation, amino acids concentrations in the fermentation broth were determined (Fig. 6C). The L-isoleucine production of IDW102 (3.81 g/L) was 8% higher, whereas the L-lysine production (0.97 g/L) was 30% lower than those of IDW101. As for other by-products (glycine, L-alanine and L-glutamate), a 52% decrease in L-alanine accumulation was observed in IDW102 compared with IDW101. These results suggested that (i) deletion of *ddh* could attenuate L-lysine by-production in an L-isoleucine production background; (ii) the attenuated carbon flux could be partially forced into L-isoleucine biosynthesis and thus slightly enhance L-isoleucine titer; (iii) deletion of *ddh* could significantly boost the cell growth of the L-isoleucine producing *C. glutamicum*.

IDW103 exhibited similar cell growth and glucose consumption pattern to IDW102, during batch fermentation (Fig. 6A, B). After 72 h cultivation, the L-isoleucine production of IDW103 (3.75 g/L) was comparable to that of IDW102 (3.81 g/L) (Fig. 6C). While the L-lysine production of IDW103 (0.19 g/L) was 80% lower than that of IDW102. As for other by-products (glycine, L-alanine and L-glutamate), a substantial change in L-glutamate accumulation was observed between IDW103 and IDW102. The L-glutamate accumulation of IDW103 (1.26 g/L) was approximately 10 folds of that of IDW102 (Fig. 6C). In contrast, IDW104 exhibited similar cell growth pattern to IDW101, whereas its L-isoleucine production was even slightly lower than that of IDW101. As for by-products, IDW104 produced 78% lower L-lysine but 1.3-fold higher L-alanine in comparison to IDW101, The substantially reduced L-lysine accumulation, the almost unchanged L-isoleucine production and the significantly increased L-glutamate or L-alanine accumulation were all indicative of a metabolic redistribution caused by *lysE* deletion in the L-isoleucine production background. So far, metabolic engineering in *C. glutamicum* for L-isoleucine production has been focused on enhancing the carbon flux in the L-isoleucine terminal pathway [11,16,19,36], strengthening L-isoleucine export [37,38] and enhancing the NADPH availability [39–41]. The different effects of *lysE* deletion on L-threonine and L-isoleucine production (Figs. 5 C, 6 C) observed here indicated that potential bottlenecks hampering L-isoleucine production might exist elsewhere in the metabolic network, which remains to be discovered.

4. Conclusions

In this work, a basal strain with eliminated L-lysine accumulation was constructed from the wild-type *C. glutamicum* ATCC13869, and further engineered into L-threonine and L-isoleucine producers. Combinotorial deletion of *ddh* and *lysE* was demonstrated as an effective solution to reducing L-lysine accumulation while maintaining the robustness of the strains. It should be noted that regardless of a wild-type background, an L-threonine production background or an L-isoleucine production background, the strains with ddh deletion maintained relatively lower intracellular L-lysine levels (Fig. 7). Furthermore, deletion of *ddh* and *lysE* was found to be effective for enhancing L-threonine production in *C. glutamicum*, and deletion of *ddh* was found to be effective for improving the cell growth of L-isoleucine producing *C. glutamicum*. The obtained L-threonine production strain TDW103 and L-isoleucine production strain IDW103, both with little L-lysine by-production, could serve as starting strains for further metabolic engineering to achieve high L-threonine and L-isoleucine titers.

Author agreement

All authors have seen and approved the final version of the manuscript being submitted. I would like to declare on behalf of my co-authors that the work described in the manuscript was original research that has not been published previously, and not under consideration for publication elsewhere.

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