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Rational modification of the carbon metabolism of *Corynebacterium glutamicum* to enhance L-leucine production

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Abstract

L-Leucine is an essential amino acid that has wide and expanding applications in the industry. It is currently fast-growing market demand that provides a powerful impetus to further increase its bioconversion productivity and production stability. In this study, we rationally engineered the metabolic flux from pyruvate to L-leucine synthesis in *Corynebacterium glutamicum* to enhance both pyruvate availability and L-leucine synthesis. First, the *pyc* (encoding pyruvate carboxylase) and *avtA* (encoding alanine-valine aminotransferase) genes were deleted to weaken the metabolic flux of the tricarboxylic acid cycle and reduce the competitive consumption of pyruvate. Next, the transcriptional level of the *alaT* gene (encoding alanine aminotransferase) was down regulated by inserting a terminator to balance L-leucine production and cell growth. Subsequently, the genes involved in L-leucine biosynthesis were overexpressed by replacing the native promoters P_{leuA} and P_{ilvBNC} of the *leuA* gene and *ilvBNC* operon, respectively, with the promoter P_{tuf} of *eftu* (encoding elongation factor Tu) and using a shuttle expression vector. The resulting strain WL-14 produced 28.47 ± 0.36 g/L L-leucine in shake flask fermentation.

Keywords L-Leucine · *Corynebacterium glutamicum* · Metabolic engineering · Attenuation · Promoter

Introduction

L-Leucine, one of the branched-chain amino acids (BCAAs), is an essential amino acid that plays important roles in a variety of biological processes [1]. It is currently widely used in industries as a flavor enhancer, an animal feed additive, an ingredient in cosmetics, and a specialty nutrient in pharmaceutical and medical fields [1, 2]. It has recently attracted much research attention because of its potential in improving human health and its fast-growing market demand [3]. Traditional extraction of L-leucine from protein hydrolysate and enzymatic synthesis has been replaced by microbial production in the industry [1]. *Corynebacterium glutamicum* has been recognized as a generally regarded as safe (GRAS) strain for the production of most amino acids, particularly L-glutamate, L-lysine, and BCAAs [4]. Due to the long biosynthetic pathway of L-leucine and the complex regulatory mechanism, the yield and conversion rate of L-leucine are still low in microbial strains [5]. Therefore, the requirement is to culture strains with high production capacity, high conversion rate, and stable production performance.

The biosynthetic pathway of BCAAs has been relatively clearly established; in *C. glutamicum*, their biosynthesis is controlled at the transcriptional level through a complex regulatory cascade [6–8]. The four common enzymes that use pyruvate as a precursor and are involved in parallel reactions are acetohydroxyacid synthase (AHAS), acetohydroxyacid isomeroreductase (AHAIR), dihydroxyacid dehydratase (DHAD), and branched-chain amino acid transaminase (BCAT) (Fig. 1). AHAS is the key enzyme of BCAA biosynthetic pathways. L-Leucine is derived from α-ketoisovalerate, a direct precursor of L-valine, in a specific series of four reactions [9]. Additionally, α-isopropylmalate synthase (IPMS), as a rate-limiting enzyme, catalyzes the conversion of α-ketoisovalerate and acetyl-CoA to α-isopropylmalate.

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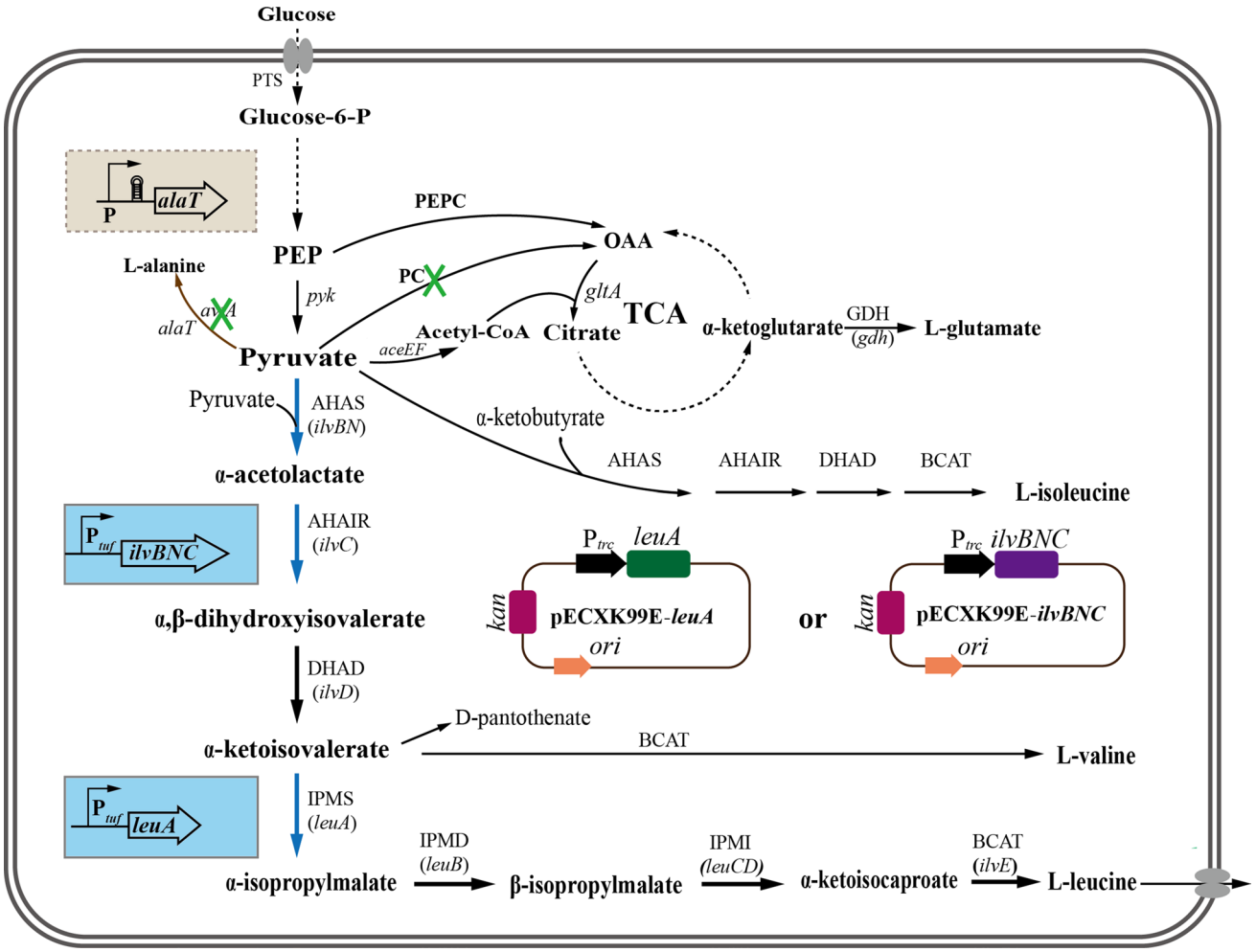

Fig. 1 The biosynthetic pathways of L-leucine in *C. glutamicum* and the strategies for constructing L-leucine-producing strains. The blue boxes represented the genes that were upregulated by promoter replacement in the genome. The brown box represented the gene that was downregulated by terminator insertion in the genome. The cross marks represented the genes that were deleted. The *ilvBNC* and *leuA* genes were overexpressed using the expression vector pEC-XK99E. *TCA* tricarboxylic acid cycle, *PEP* phosphoenolpyruvate, *OAA* oxaloacetate, *PEPC* phosphoenolpyruvate carboxylase, *PC* pyruvate carboxylase

Development of bacterial strains for L-leucine production has not yet been successful, primarily because they possess complicated regulatory mechanisms for BCAA synthesis [10, 11].

Pyruvate is the end product of glycolysis, and it enters into the tricarboxylic acid (TCA) cycle [12]. Several studies have revealed that pyruvate availability is critical for pyruvate-amino acid overproduction [13, 14]. However, an active TCA cycle is not conducive to the efficient synthesis of pyruvate-family amino acids, because it leads to a waste of carbon [15]. Hence, there may be a trade-off between the TCA cycle and product synthesis. Additionally, the phosphoenolpyruvate (PEP)–pyruvate–oxaloacetate (OAA) node is one of the most important links between glycolysis and the TCA cycle [16]. A series of reactions at this node could direct the carbon flux into appropriate directions, making it a highly relevant switch point for carbon flux distribution within the central metabolism [16, 17]. In this node, phosphoenolpyruvate carboxylase (PEPC) catalyzes the conversion of PEP to OAA, and pyruvate carboxylase (PC) catalyzes the conversion of pyruvate to OAA [17]. PEPC and PC are two important anaplerotic enzymes for supplying OAA in *C. glutamicum*, and OAA supply determines the flux of the TCA cycle [18]. The metabolic flow of the PEP–pyruvate–OAA node may be significantly changed by regulating the TCA cycle supplementation pathway that PEPC and PC participate in [7, 19].

In *C. glutamicum*, L-alanine, one of the main by-products of L-leucine production, is biosynthesized from pyruvate via two distinct pathways [20]. Alanine aminotransferase (AlaT) converts pyruvate to L-alanine in an L-glutamate-dependent manner, and alanine-valine aminotransferase (AvtA)

converts pyruvate to L-alanine in an L-valine-dependent reaction [21, 22]. *C. glutamicum* with a deletion of either of the corresponding genes does not exhibit an obvious growth deficiency, but a double mutant is auxotrophic for L-alanine; this suggests that AlaT and AvtA are the only aminotransferases involved in L-alanine biosynthesis and they cannot be deleted simultaneously [23]. Although the addition of L-alanine to the medium might recover cell growth, it would likely incur additional costs. Thus, the attenuation of *alaT* expression is a potential strategy for balancing L-leucine yield and cell growth. Transcription is the first stage of gene expression, and the terminator is an important element of the transcription process and thus crucial for protein expression [24, 25]. Terminators may be used as a tuning knob to down regulate *alaT* expression and improve L-leucine production.

Alteration of the expression of some important genes via promoter replacement is a widely used approach to enhance production. At the same time, it should be noted that in addition to increasing the expression of certain genes, the precise control of gene expression is a critical step, which can influence the amount of key pathway enzymes available for maximizing the production of target products [26, 27]. This objective can be achieved using promoters with different strengths. Promoters P_{tac} and P_{tuf} with different strengths are widely used to improve the expression level of enzymes in *C. glutamicum* [1, 20, 22, 28, 29].

A few of the current strain development strategies, including elimination of the feedback inhibition of enzymes [4, 22], strengthening carbon influx by overexpressing the key genes and weakening the competing branches [1, 22], optimizing the aminotransferases to increase enzyme specificity [2], and altering the redox balance [6], have yielded notably improved L-leucine-producing strains. Additionally, high production efficiency is a crucial constraint for any economically viable industrial production process [30, 31]. In this study, the *pyc* gene (encoding PC) was deleted to regulate the TCA cycle supplementation pathway and increase the pyruvate availability. Subsequently, the *avtA* gene was deleted and the *alaT* expression was downregulated to weaken the metabolic flux from pyruvate to L-alanine. Considering the importance of *ilvBNC* and *leuA* in the L-leucine biosynthetic pathway, two promoters with differing strengths, viz., P_{tuf} and P_{tac}, were selected to fine-tune the expression of *ilvBNC* and *leuA*, respectively.

Materials and methods

Bacterial strains, plasmids, and culture conditions

The strains and plasmids used in this study are listed in Table 1. The L-leucine-producing strain *C. glutamicum* XQ-9-Δ*ltbR*, which was derived from wild-type *C. glutamicum* ATCC 13,032 and stored in our laboratory, was used as the working and parent strain [6]. This strain was auxotrophic for L-isoleucine and L-methionine. Many amino acid exchanges occur in *C. glutamicum* XQ-9-Δ*ltbR* with respect to the corresponding sequence from the wild-type strain ATCC 13,032 [6]. Especially, there are five amino acid exchanges related to the *leuA* gene, suggesting that the working strain XQ-9-Δ*ltbR* with feedback-resistant enzymes could accumulate L-leucine, whereas the wild-type strain typically would not *Escherichia. coli* JM109 and BL21 (DE3) were grown in LB medium (5-g/L yeast extract, 10-g/L tryptone, and 10-g/L NaCl) at 37 °C, and *C. glutamicum* strains were grown at 30 °C in LBG (LB medium supplemented with 5-g/L glucose). LBHIS medium (5-g/L tryptone, 5-g/L NaCl, 2.5-g/L yeast extract, 18.5-g/L Brain Heart Infusion powder, and 91-g/L sorbitol) was used for the transformation of the mutant gene into *C. glutamicum* cells. Where appropriate, kanamycin and isopropyl β-D-thiogalactoside at final concentrations of 50 μg/mL and 1 mmol/L, respectively, were added to the medium.

For L-leucine production in shake flask cultivation, the strains were grown in 500-mL Erlenmeyer flasks containing 50-mL seed medium for 14–18 h [6]. The fermentation medium was inoculated with a seed solution of 10% inoculum. All strains were cultured at 30 ℃ under continuous shaking at 100 rpm. The fermentation lasted for 72 h. The medium used for seed culture contained 30 g/L glucose, 35 g/L corn steep liquor, 5 g/L $(NH_4)_2SO_4$, 1.3 g/L $KH_2PO_4{\cdot}3H_2O$, 0.4 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $MnSO4{\cdot}H_2O$, 10 g/L sodium citrate, 10 g/L yeast extract, 2 g/L urea, 0.4 g/L L-methionine, 0.0002 g/L biotin, 0.0003 g/L thiamine, and 20 g/L $CaCO_3$. The fermentation medium contained 130 g/L glucose, 25 g/L corn steep liquor, 15 g/L $(NH_4)_2SO_4$, 15 g/L CH_3COONH_4, 1.3 g/L $KH_2PO_4{\cdot}3H_2O$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.01 g/L $MnSO_4{\cdot}H_2O$, 2 g/L sodium citrate, 2 g/L urea, 0.8 g/L L-methionine, 0.06 g/L L-isoleucine, 0.5 g/L L-glutamate, 0.00008 g/L biotin, 0.0002 g/L thiamine, and 30 g/L $CaCO_3$. Both media were adjusted to pH 7.2 with NaOH.

Recombinant DNA work for plasmid and strain construction

All DNA primers were synthesized by General Biosystems Co. Ltd. (Anhui, China), and are listed in Table S1. The shuttle vector pEC-XK99E was used for gene transfer between *E. coli* and *C. glutamicum* and for gene expressions in *C. glutamicum* [6]. The vector pK18*mobsacB* was used for in-frame deletions and integrations of DNA sequences via two-step homologous recombination in *C. glutamicum* [22]. The upstream and downstream fragments of the *pyc* gene were amplified from the genome of *C. glutamicum* XQ-9-Δ*ltbR*, and then these fragments were overlapped

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
JM109	recA1 end1 gyrA96 thi hsdR17 supE44 relA1 Δ(lac-proAB)/F'(traD36 proAB$^+$ lac^q lacZ ΔM15)	Lab stock
BL21(DE3)	F- ompT gal dcm lon hsdS_B (r_B- m_B-) λ(DE3)	Lab stock
C. glutamicum		
Wild type	Wild-type ATCC 13,032, biotin auxotrophic	ATCC
ΔLtbR	*C. glutamicum* XQ-9 derivative with in-frame deletion of *ltbR*	Lab stock
WL-1	ΔLtbR derivative with in-frame deletion of *pyc*	This study
WL-2	ΔLtbR derivative with in-frame deletion of *ppc*	This study
WL-3	WL-1 derivative with in-frame deletion of *ppc*	This study
WL-4	WL-2 derivative with in-frame deletion of *avtA*	This study
WL-5	WL-4 derivative with in-frame deletion of *alaT*	This study
WL-6	WL-4 derivative with insertion of terminator T1 in front of *alaT*	This study
WL-7	WL-4 derivative with insertion of terminator T2 in front of *alaT*	This study
WL-8	WL-4 derivative with insertion of terminator T3 in front of *alaT*	This study
WL-9	WL-4 derivative with insertion of terminator T4 in front of *alaT*	This study
WL-10	WL-8 derivative with P_{tac} promoter inserted in front of *ilvBNC* and *leuA*, respectively	This study
WL-11	WL-8 derivative with P_{tac} promoter inserted in front of *ilvBNC* and P_{tuf} promoter inserted in front of *leuA*	This study
WL-12	WL-8 derivative with P_{tuf} promoter inserted in front of *ilvBNC* and *leuA*, respectively	This study
WL-13	WL-12 harboring vector pECXK99E	This study
WL-14	WL-12 harboring vector pEC-*leuA*	This study
WL-15	WL-12 harboring vector pEC-*ilvBNC*	This study
Plasmids		
pECXK99E	Kan^r, *E. coli-C. glutamicum* shuttle vector for inducible gene expression	Lab stock
pDXW-8	Kan^r, *E. coli-C. glutamicum* shuttle vector for inducible gene expression (P_{tac}, lacI,)	Lab stock
pK18*mobsacB*	Kan^r, intergration vector; $oriV_{Ec}$ *oriT sacB*, allows for selection of double crossover *C. glutamicum*	Lab stock
pEC-*leuA*	Kan^r, pECXK99E derivative containing gene *leuA* (*C. glutamicum* XQ-9 Δ*ltbR*)	This study
pEC-*ilvBNC*	Kan^r, pECXK99E derivative containing gene *ilvBNC* (*C. glutamicum* XQ-9 Δ*ltbR*)	This study
pK18-Δ*pyc*	Kan^r, pK18*mobsacB* derivative for in-frame deletion of gene *pyc*	This study
pK18-Δ*ppc*	Kan^r, pK18*mobsacB* derivative for in-frame deletion of gene *ltbR*	This study
pK18-Δ*avtA*	Kan^r, pK18*mobsacB* derivative for in-frame deletion of gene *avtA*	This study
pK18-Δ*alaT*	Kan^r, pK18*mobsacB* derivative for in-frame deletion of gene *alaT*	This study
pK18-T1	Kan^r, pK18*mobsacB* derivative containing the fragment of insertion T1 terminator in front of *alaT*	This study
pK18-T2	Kan^r, pK18*mobsacB* derivative containing the fragment of insertion T2 terminator in front of *alaT*	This study
pK18-T3	Kan^r, pK18*mobsacB* derivative containing the fragment of insertion T3 terminator in front of *alaT*	This study
pK18-T4	Kan^r, pK18*mobsacB* derivative containing the fragment of insertion T4 terminator in front of *alaT*	This study
pK18*mobsacB*-P_{tac}-*leuA*	Kan^r, pK18*mobsacB* derivative containing *leuA* gene under control of the P_{tac} promoter along with 100 bp	This study
pK18*mobsacB*-P_{tac}-ilv*BNC*	Kan^r, pK18*mobsacB* derivative containing *ilvBNC* gene under control of the P_{tac} promoter along with 100 bp	This study
pK18*mobsacB*-P_{tuf}-*leuA*	Kan^r, pK18*mobsacB* derivative containing *leuA* gene under control of the P_{tuf} promoter along with 200 bp	This study
pK18*mobsacB*-P_{tuf}-ilv*BNC*	Kan^r, pK18*mobsacB* derivative containing *ilvBNC* gene under control of the P_{tuf} promoter along with 200 bp	This study

together by PCR, resulting in pK18*mobsacB*-Δ*pyc*. The upstream and downstream fragments of the *ppc* gene (encoding PEPC) were also amplified from the genome of *C. glutamicum* XQ-9-Δ*ltbR* and then assembled with the *Eco*RI/*Hind*III-linearized pK18*mobsacB* fragment using a seamless cloning kit, resulting in pK18*mobsacB*-Δ*ppc*. The same method was applied to construct pK18*mobsacB*-Δ*avtA* and pK18*mobsacB*-Δ*alaT* using corresponding primer pairs. Moreover, for inserting the terminator in front of the *alaT* gene, four terminator sequences with strengths of 10.94 a.u., 29.97 a.u., 40.39 a.u., and 93.18 a.u., discovered and measured by Chen et al. [25], were added between the upstream and downstream primer sequences. Subsequently, the PCR products were assembled with the *Eco*RI/*Bam*HI-linearized pK18*mobsacB* fragment using a seamless cloning kit. In addition, for promoter replacement in the genome, the upstream and downstream fragments and the promoter sequence fragment were assembled into plasmid pK18*mobsacB*. Promoter P_{tac} and P_{tuf} sequences were amplified using pDXW-8 and *C. glutamicum* ATCC 13,032 genome as template, respectively. Finally, as per the method described in a previous study [6], the *leuA* and *ilvBNC* genes were cloned into plasmid pEC-XK99E. For strain construction, plasmids were transformed into *C. glutamicum* by electroporation. All constructed plasmids including chromosomal deletions and integrations in the engineered strains were verified by DNA sequencing.

RT-PCR for mRNA quantification

Total RNA was isolated from all bacterial strains using the EZ-10 Total RNA Mini-Preps Kit (Sangon Biotech, Shanghai, China) and was then used as the template to synthesize cDNA following the AMV reverse transcriptase-based protocol (Sangon Biotech, Shanghai, China). The cDNA targets were quantified with the PrimeScript™ One Step RT-PCR Kit II (Takara, Tokyo, Japan) using 16S RNA as the internal control [32]. PCR primers used in RT-PCR are listed in Table S1.

Analytical methods

Samples were taken every 12 h to determine various parameters. Cell growth was monitored by measuring the optical density of the culture at 562 nm (OD_{562}) using a spectrophotometer (721 N, Shanghai, China) after diluting 0.2 mL of the samples with 5 mL of 0.25 M HCl to dissolve $CaCO_3$. The dry cell weight (DCW) per liter was calculated using the formula: $DCW\ (g/L) = 0.57 \times OD_{562} + 0.23$, which was experimentally determined in a previous study [21]. Glucose and glutamate concentrations were analyzed using the SBA-40E immobilized enzyme biosensor (Biology Institute of Shandong Academy of Sciences, Jinan, China). Pyruvate compounds (L-leucine, L-valine, and L-alanine) were analyzed by high-pressure liquid chromatography (HPLC) on an Agilent 1200 system (Agilent Technologies, Santa Clara, CA, USA) [6].

Results and discussion

Increasing cytoplasmic pyruvate availability by deletion of the *pyc* gene

Recently, precursor supplementation has emerged as an attractive strategy to improve the production of the corresponding metabolite [33]. L-leucine is produced from pyruvate, and the pyruvate node comprises a series of metabolic pathways, including L-glutamate, L-alanine, and other BCAA biosynthetic pathways, that compete for carbon fluxes with L-leucine synthesis. Thus, pyruvate availability is already recognized as a major bottleneck for L-leucine production [33]. It is well known that the PEP–pyruvate–OAA node plays an important role in amino acid synthesis [7]. Therefore, deletion of the genes *pyc* (encoding PC) and/or *ppc* (encoding PEPC) involved in the anaplerotic pathway would be expected to further regulate the carbon flux distribution at the pyruvate node so as to decrease the production of by-products involved in the TCA cycle. As shown in Fig. 2a, the PC-deficient strain WL-1 (*pyc* gene deletion) produced 16.86 ± 0.50 g/L of L-leucine, which was approximately 7% higher than the amount of L-leucine produced by the original strain ΔLtbR (15.72 ± 0.72 g/L). PEPC is dispensable. Several studies have revealed that the PC-catalyzed anaplerotic reaction plays an important role in glutamate production, and PEPC activity in *C. glutamicum* is distinctly enhanced under PC deficiency [18, 19]. Enhanced L-leucine production in the WL-1 strain in our study suggested that the supplementation of OAA, which is catalyzed only by PEPC, could not maintain the original metabolic flux of the TCA cycle, and instead, the flux from pyruvate to α-acetolactate was enhanced. In addition, L-valine production by the WL-1 strain was also increased (from 5.93 ± 0.27–6.74 ± 0.19 g/L) (Fig. 2d). We consider this as a consequence of shared enzymes and intermediates in the synthesis of BCAAs. However, the PEPC-deficient strain WL-2 (*ppc* gene deletion) produced only 8.13 ± 0.39 g/L of L-leucine but led to L-glutamate accumulation of up to 4.24 ± 0.22 g/L, which was much higher than that by the ΔLtbR strain (Fig. 2a, b). It was probable that the supplementation of OAA, which is believed to form a precursor of L-glutamate, was increased, because PC activity could be enhanced under the PEPC-deficient condition. The intracellular concentration of pyruvate decreased because of the improved PC activity, which in turn decreased the conversion of pyruvate to other

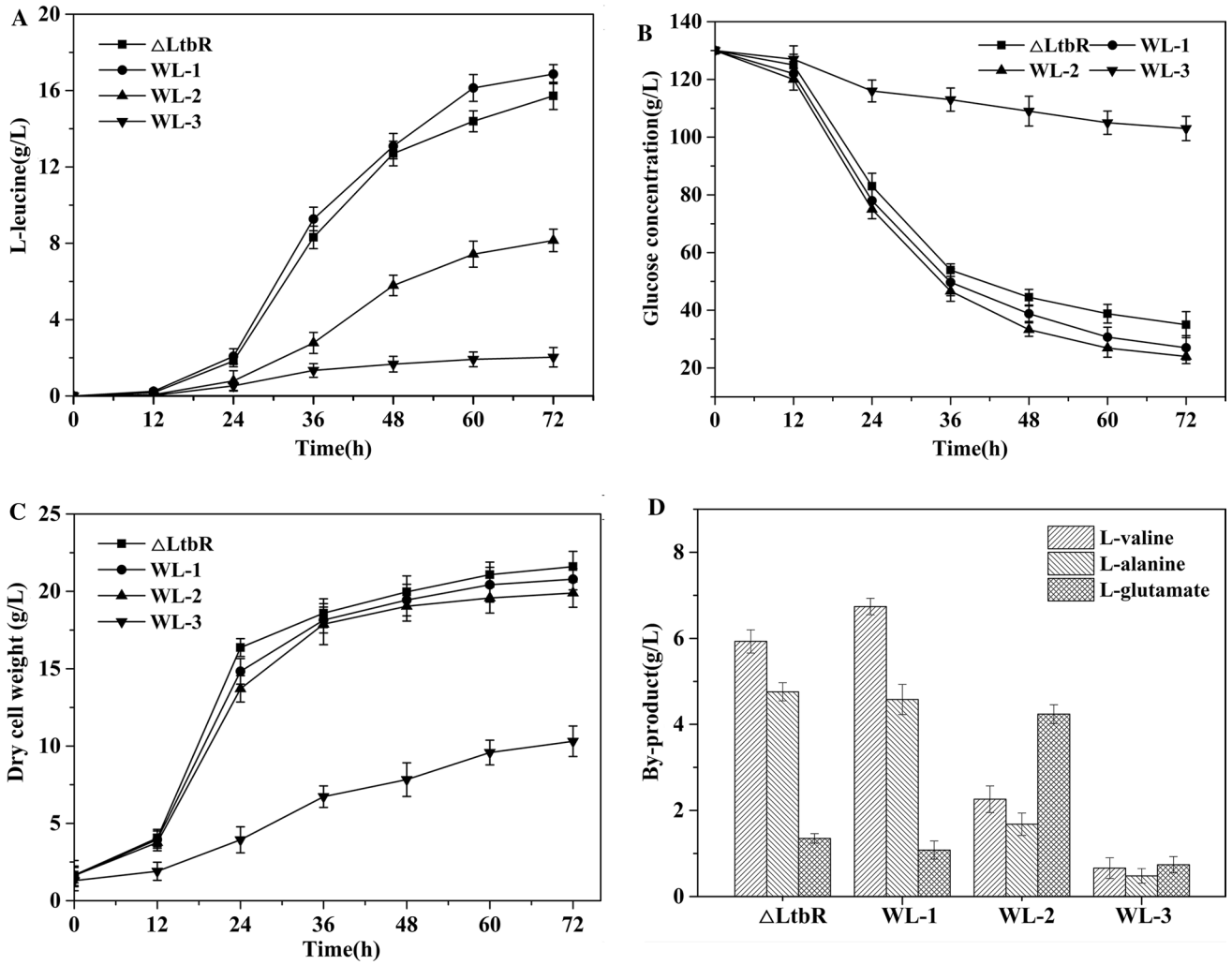

Fig. 2 Comparison of different *C. glutamicum* strains during shake flask cultivation in fermentation medium to test the effects of *pyc* and *ppc* deletion. **a** L-Leucine production, **b** glucose concentration, **c** cell growth, and **d** by-product accumulation. All strains were tested in triplicate. The error bar denotes standard deviation of the mean

metabolites such as BCAAs. Interestingly, the cell growth remained almost unaffected, whereas glucose consumption was slightly increased by the inactivation of PC or PEPC (Fig. 2b, c). These results indicate that PC disruption had a positive impact on L-leucine production, whereas PEPC disruption was conductive to L-glutamate synthesis. Thus, we conclude that adequate OAA supplementation in the WL-1 strain was mainly provided by the PC reaction, and the *pyc* gene deletion could downregulate the metabolic flux of the TCA cycle, which facilitated L-leucine synthesis. This conclusion is consistent with previous studies showing that improving the intracellular availability of precursor reduces carbon flux to undesirable by-products and increases the yield of the target products [34, 35]. Furthermore, cutting off either PEPC or PC did not affect the growth of *C. glutamicum*. This result was consistent with a previous study showing that these two enzymes can compensate for the loss of the other [36]. Notably, double disruption of PEPC and PC (i.e., the WL-3 strain) led to an explicit growth deficiency and almost no L-leucine yield (Fig. 2a, c).

Decreasing L-alanine accumulation by deletion of *avtA* gene and attenuation of alaT expression

The above-mentioned results showed that L-valine and L-alanine were the main by-products of L-leucine production by fermentation, which had a negative impact on the yield and downstream processing [22, 37]. Due to the tightly coordinated regulatory mechanism of BCAA biosynthesis, it is infeasible to directly delete *ilvE* to block L-valine biosynthesis by traditional methods [2, 38]. Moreover, in *C. glutamicum*, L-alanine biosynthesis occurs via two pathways, one catalyzed by AlaT and another by AvtA, with pyruvate used as the direct precursor [39], and the former pathway

is the major contributor [40]. To decrease the competitive consumption of pyruvate and the conversion of L-valine to L-alanine, *avtA* was first knocked out in *C. glutamicum* WL-1, resulting in the WL-4 (WL-1-Δ*avtA*) strain. Compared with the WL-1 strain, the WL-4 strain showed slightly higher L-valine production, but no significant change in L-leucine production (Figs. 2d, 3). Additionally, alanine accumulation by WL-4 was slightly lower than that by WL-1 (4.63 ± 0.13 g/L vs. 5.27 ± 0.35 g/L), showing that L-alanine accumulation is achieved only by the *alaT*-catalyzed reaction. These results also indicate that the *avtA* gene knockout was effective for decreasing pyruvate consumption and L-alanine accumulation. However, compared with the WL-4 strain, the L-leucine production and cell growth rate of the double mutant WL-5 strain were both dramatically reduced (Fig. 3). These results are consistent with a previous study reporting that the double mutant produces some undesired results, including extra nutritional requirements and growth deficiency [21]. With the aim of further decreasing L-alanine production in the WL-4 strain, attenuation of *alaT* expression was a potential strategy that also balanced cell growth and L-leucine production [41]. Thus, four terminator sequences, discovered by Chen et al. [25] (Table 2), with strengths of 10.94 a.u., 29.97 a.u., 40.39 a.u., and 93.18 a.u., were selected and inserted in front of the *alaT* gene in the *C. glutamicum* genome, resulting in WL-6, WL-7, WL-8, and WL-9 mutants, respectively. Fermentation experiments were then performed to evaluate the effects of these modifications on cell growth and amino acid production. As shown in (Fig. 3), these four recombinant strains with different terminator strengths had different L-leucine yields as compared with the WL-4 strain. The L-leucine yield of the WL-8 strain was 18.13 ± 0.34 g/L, which was 6% higher than that of the WL-4 strain (17.08 ± 0.53 g/L). Furthermore, L-alanine accumulation by the WL-8 strain was 3.48 ± 0.21 g/L, which was 25% lower than that by the WL-4 strain (4.63 ± 0.13 g/L). As shown in Fig. 3, there was a remarkable improvement in the cell growth of the WL-6, WL-7, and WL-8 strains compared with the control strain WL-5. These results suggested that *alaT* expression was downregulated by inserting a terminator, resulting in the slight improvement of L-leucine production. Compared with the WL-4 strain; however, L-leucine production of the WL-9 strain decreased by 43% (Fig. 3). These results suggest that the insertion of a terminator with higher strength also affected the cell growth. Thus, in consideration of both cell growth and L-leucine production, the terminator with the strength of 40.39 a.u. was considered to be optimal for downregulating *alaT* expression. The optimal strain WL-8 relieved the cell growth disturbance and weakened L-alanine accumulation. Consequently, *C. glutamicum* WL-8 was used for the subsequent experiments.

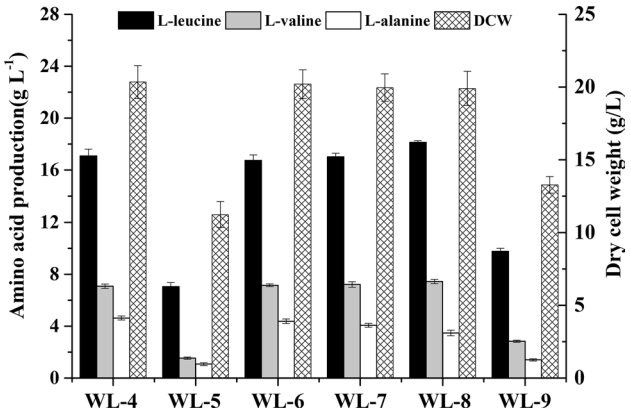

Fig. 3 Comparison of different *C. glutamicum* strains during shake flask cultivation in fermentation medium to test the effect of decreasing L-alanine accumulation by attenuating *alaT* expression. Cell growth and amino acid (L-leucine, L-valine, and L-alanine) productions of the strains

Balancing the *ilvBNC* and *leuA* expression levels by inserting promoters with different strengths to improve L-leucine accumulation

The ultimate aim of this study was to maximize L-leucine production in *C. glutamicum*. AHAS catalyzes the key reaction in BCAA biosynthesis, and the *ilvBNC* operon plays a vital role in the BCAA biosynthetic pathways [11, 42]. α-Ketoisovalerate, the last intermediate of L-valine synthesis, is also the precursor for L-leucine catalyzed by IPMS [43]. Many reports have confirmed that *ilvBNC* and *leuA* overexpression promote L-leucine accumulation [6, 44]. Promoters are important elements in the transcriptional regulation of cells, and precisely regulating gene transcription by engineering promoters has become a very important metabolic engineering strategy [28, 34, 45]. The replacement of

Table 2 Terminator sequence and strength

Name	Sequence (5′-3′)	Strength (a.u.)
Terminator T1	ACGAGCCAATAAAAATACCGGCGTTATGCCGGTATTTTTTTACGAAAGA	10.94
Terminator T2	GGGCGGTCAGATGATCGCCCTTTTTTTTT	29.97
Terminator T3	AAGACCCCCGCACCGAAAGGTCCGGGGGTTTTTTTT	40.39
Terminator T4	CCAATTATTGAACACCCAAATCGGGTGTTTTTTTGTTTCTGGTCTCCC	93.18

original promoters with promoters of various strengths is an effective strategy to fine-tune gene expression. Hence, for precisely regulating gene transcription and achieving a balance of carbon metabolism, two promoters with differing strengths, viz., P_{tac} and P_{tuf}, were used to rationally replace the native promoters of *leuA* and *ilvBNC* in the ΔLtbR strain, resulting in WL-10, WL-11, and WL-12 strains (Fig. 4f). In the WL-10 strain, the native promoters of *ilvBNC* and *leuA* in the genome were replaced by the strong P_{tac} promoter to enhance L-leucine biosynthesis. Gene expressions of *ilvB*, *ilvN*, *ilvC*, and *leuA* at 16 h during fermentation were tested by qRT-PCR, and the results showed that the promoter replacement strategy increased the translational levels of *ilvB*, *ilvN*, *ilvC*, and *leuA* (Fig. 4e). Data analysis indicated that compared with the WL-8 strain, the translational level of *leuA* in the WL-10 strain was increased by two times and that of *ilvB*, *ilvN*, and *ilvC* was increased by > 1.5 times. After fermentation assay, WL-10 produced 22.38 ± 0.53 g/L of L-leucine, which was 23% higher than the L-leucine yield of WL-8 (18.13 ± 0.34 g/L) (Fig. 4a). Moreover, glucose consumption and cell growth of WL-10 were improved slightly (Fig. 4b, c). Additionally, the accumulation of main by-products by WL-10 was remarkably lower than that by WL-8. These findings indicate that promoter replacement with the strong P_{tac} promoter enhanced the expression of genes involved in L-leucine synthesis, and more pyruvate was thus available for use in L-leucine synthesis. In addition, the production of by-products decreased. Similar results were also obtained with the other recombinant strains WL-11 and WL-12. Notably, *leuA* expression increased by 2.6-fold in the WL-11 strain (native promoters of *ilvBNC* and *leuA* replaced with P_{tac} promoter and P_{tuf} promoter, respectively) compared with that in the WL-8 strain, resulting in 24.73 ± 0.34-g/L yield of L-leucine. Meanwhile, L-valine accumulation by the WL-11 strain reduced to 3.26 ± 0.19 g/L, which was approximately 40% lower than that by the WL-10 strain (Fig. 4a, d). Therefore, *leuA* overexpression by the insertion of P_{tuf} promoter more effectively increased the carbon flux into the L-leucine biosynthetic pathway and reduced L-valine accumulation. This finding is consistent with the previous result reported by Vogt et al. [22]. Notably, the WL-12 strain (native promoters of both *ilvBNC* and *leuA* replaced with P_{tuf} promoter) provided the highest L-leucine yield (26.83 ± 0.42 g/L) among the three recombinant strains (WL-10, WL-11, and WL-12). The transcriptional levels of *ilvB*, *ilvN*, and *ilvC* in WL-12 were upregulated by > 1.9-fold compared with those in WL-8 (Fig. 4a, e). Furthermore, L-valine production by WL-12 was 4.58 ± 0.25 g/L, which was higher than that by WL-11 (Fig. 4d). This finding is attributable to the fact that leucine synthesis competes with the synthesis of other BCAAs for common precursors and the same enzymes [6]. Moreover, the insertion of P_{tuf} promoter in front of *ilvBNC* and *leuA* in the WL-12 strain more effectively increased carbon flux into

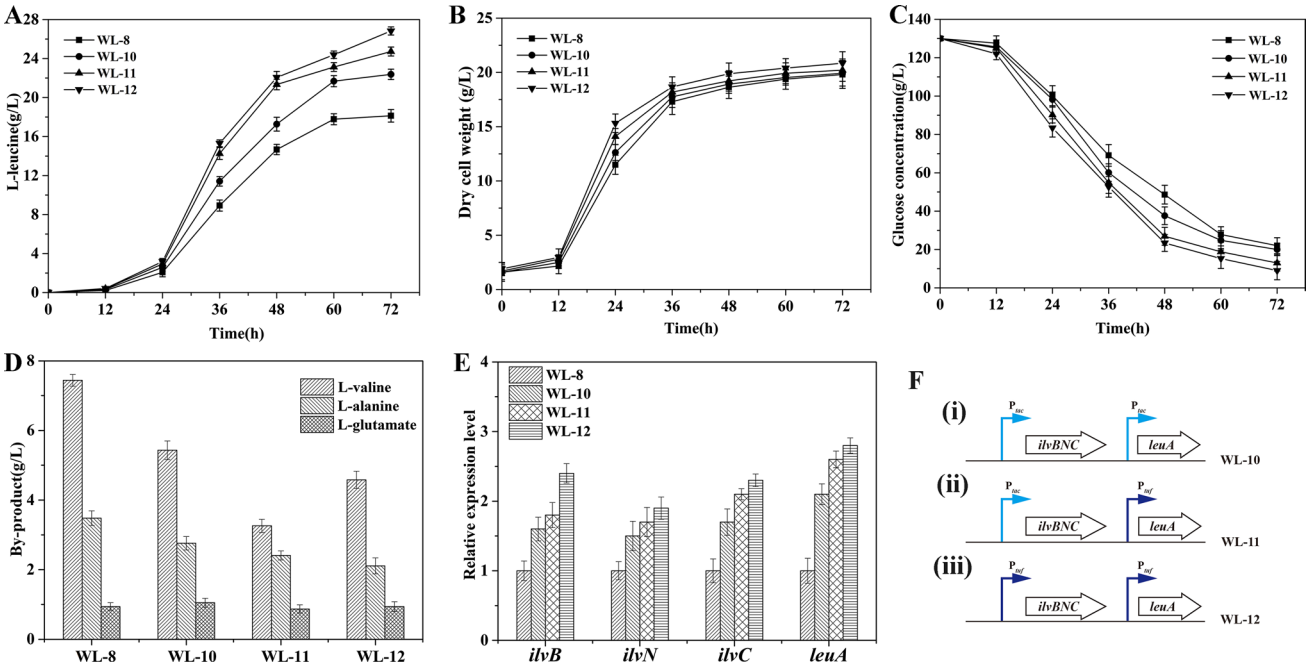

Fig. 4 Comparison of different *C. glutamicum* strains during shake flask cultivation in fermentation medium to test the effect of balancing *ilvBNC* and *leuA* expression levels by inserting promoters of differing strengths. **a** L-Leucine production, **b** glucose concentration, **c** cell growth, **d** by-product accumulation, **e** transcriptional levels of L-leucine biosynthesis genes, and **f** combination of strong promoter replacement

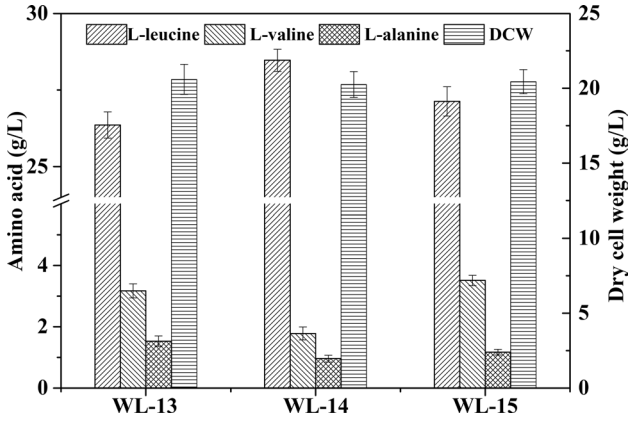

Fig. 5 Comparison of different *C. glutamicum* strains during shake flask cultivation in fermentation medium to test the effect of further enhancing *ilvBNC* and *leuA* overexpression. Cell growth and amino acid (L-leucine, L-valine, and L-alanine) productions of the strains

L-leucine synthesis, in addition to increasing L-valine production. In summary, the WL-11 strain provided the lowest L-valine yield (3.26 ± 0.19 g/L) among the three recombinant strains, whereas the WL-12 strain provided the highest L-leucine yield (26.83 ± 0.42 g/L).

Further enhancing *ilvBNC* and *leuA* overexpression using a shuttle expression vector to improve L-leucine accumulation

To further enhance L-leucine synthesis, the key genes involved in L-leucine synthesis were overexpressed using the shuttle expression vector pEC-XK99E in the WL-12 strain. The WL-12 strain carrying an empty plasmid was used as the control (i.e., the WL-13 strain). As expected, the L-leucine yield reached 28.47 ± 0.36 g/L and 27.13 ± 0.48 g/L in the WL-14 with *leuA* expression and WL-15 with *ilvBNC* expression strains, respectively. L-valine accumulation in the WL-14 strain decreased to 3.85 ± 0.11 g/L (Fig. 5). These results further indicate that increasing *leuA* expression was a very efficient approach to redirect the metabolic flux from α-ketoisovalerate to L-leucine synthesis rather than L-valine synthesis. Notably, the affinity of IPMS for α-ketoisovalerate is tenfold greater than that of BCAT [9]. Hence, *leuA* overexpression was the most significant step to increase L-leucine production and reduce L-valine accumulation in *C. glutamicum*. One possible reason for L-valine accumulation and inadequate L-leucine yield could be the shortage of the second IPMS substrate acetyl-CoA.

However, it important to note that cell growth decreased slightly in all of the recombinant strains (Fig. 5). This observation is consistent with previous studies reporting that plasmid replication increases the metabolic burden of recombinant *C. glutamicum* [46]. The enhancement of pyruvate availability and enzyme activities is an efficient approach to redirect the metabolic flux to L-leucine synthesis. Our future work will focus on the metabolic engineering of strains with acetyl-CoA- and glucose-uptake systems, which have been successfully constructed for L-lysine production [47].

Conclusion

In this study, various efforts were made to increase the L-leucine yield of L-leucine-producing *C. glutamicum* strains. Increasing the precursor availability and engineering the biosynthetic pathways were shown to be critical factors for enhancing L-leucine production. The pyruvate availability was improved by deleting *pyc* and *avtA* genes and downregulating *alaT* expression. Given the importance of *ilvBNC* and *leuA* genes in L-leucine synthesis, their expression was upregulated by the insertion of strong P_{tuf} promoter and a shuttle expression vector, which enhanced carbon flux from pyruvate to L-leucine synthesis. Among all the strains constructed in this study, the WL-14 strain exhibited the highest L-leucine yield (i.e., 28.47 ± 0.36 g/L).

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Compliance with ethical standards

Conflict of interest The authors report no declarations of interest.

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