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ORIGINAL PAPER

Development of L-tryptophan production strains by defined genetic modification in *Escherichia coli*

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Abstract Construction and improvement of industrial strains play a central role in the commercial development of microbial fermentation processes. L-tryptophan producers have usually been developed by classical random mutagenesis due to its complicated metabolic network and regulatory mechanism. However, in the present study, an L-tryptophan overproducing *Escherichia coli* strain was developed by defined genetic modification methodology. Feedback inhibitions of 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase (AroF) and anthranilate synthase (TrpED) were eliminated by site-directed mutagenesis. Expression of deregulated AroF and TrpED was achieved by using a temperature-inducible expression plasmid pSV. Transcriptional regulation of trp repressor was removed by deleting *trpR*. Pathway for L-Trp degradation was removed by deleting *tnaA*. L-phenylalanine and L-tyrosine biosynthesis pathways that compete with L-tryptophan biosynthesis were blocked by deleting their critical genes (*pheA* and *tyrA*). The final engineered *E. coli* can produce 13.3 g/l of L-tryptophan. Fermentation characteristics of the engineered strains were also analyzed.

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Introduction

L-tryptophan (L-Trp) is an essential amino acid for humans and other animals. It is widely used in food, animal feed, and pharmaceutical industries [13, 21]. L-Trp production by microbial fermentation in *Escherichia coli* has been extensively studied [2, 7–9]. Azuma et al. [2] constructed a strain by repeated random mutagenesis and the production of L-Trp reached 54.6 g/l by feeding of L-Trp precursors. Recently, Dodge et al. developed another strain of *E. coli* K-12 via ethyl methane sulfonate mutagenesis. The recombinant strain produced 42 g/l L-Trp with 18% conversion ratio from glucose by optimizing the glucose feed rate [9]. Although significant progress in L-Trp production has been made, the development of L-Trp overproducing strains is all involved in multiple rounds of random mutagenesis [2, 9, 15]. Previous studies showed that random mutagenesis often produces unexpected mutations at some locations in the genome together with desirable ones. Since it is difficult to ascertain the influence of these unidentified mutations, further strain improvement would be affected. These problems widely exist in industrial strains constructed by random mutagenesis.

Recent advances in molecular genetics, especially the genetic modification technology directly on genome, greatly prompt the development of genetically defined strains. As these engineering strains have completely defined genome traits, except for the production of target products, they can also be used to understand the synthetic mechanism of target products through analysis of the metabolic pathway.

So far, *E. coli* strains constructed by completely defined genetic modification have been used for the production of L-valine [24], L-threonine [20], and L-tyrosine (L-Tyr) [18] etc. However, there is no such report on the construction of L-Trp overproducing strain yet. This situation may be connected to the complicated regulation mechanism of L-Trp biosynthesis [13]. The biosynthesis of L-Trp in *E. coli* involves three major steps: firstly, the central metabolism pathway towards the condensation of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P); secondly, the common aromatic pathway from 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) to chorismate (CHA); and finally, the L-Trp branch pathway leading to L-Trp (Fig. 1). During the L-Trp biosynthesis process, there exist different kinds of regulations, containing feedback inhibition, transcriptional regulation, product degradation, and competing pathways, etc., which makes it difficult to redirect the carbon flux towards L-Trp production.

In the present study, we attempted to introduce a series of defined genetic manipulations into *E. coli* to develop an L-Trp-producing strain based on known regulatory and metabolic information. The feedback-resistant DAHP synthase encoded by *aroF* was achieved by deleting its residue Ile11. The feedback-resistant anthranilate (ANTA) synthase encoded by *trpED* was achieved by replacing the residue Ser40 of TrpE with Phe [6, 31]. The transcriptional regulation of trp repressor and the degradation reaction of L-Trp were eliminated by knocking out their encoding genes *trpR* and *tnaA*, respectively. In addition, two competing pathways leading to the biosynthesis of L-Phe and L-Tyr were blocked by knocking out their key genes *pheA* and *tyrA*, respectively. To the best of our knowledge, this is the first report of the construction of L-Trp-producing strain completely by genetically defined modifications.

Materials and methods

Bacterial strains and plasmids

The wild-type *E. coli* W3110 was used as the parent strain for a series of gene knock-out mutants. The genes, including transcriptional repressor for trp operon (TrpR), tryptophanase (TnaA), chorismate mutase/prephenate dehydratase (PheA), and chorismate mutase/prephenate dehydrogenase (TyrA), were knocked out. Each of these mutations was designated by a number (1-Δ*trpR*, 2-Δ*tnaA*, 3-Δ*pheA*, and 4-Δ*tyrA*). A list of these strains and relevant details of their properties is described in Table 1.

Low copy number vector pSV was constructed on the basis of pACYC177 (Culture and Information Centre of Industrial Microorganisms of China Universities, CICIM) and pND707 (CICIM). A 1,354-bp DNA fragment containing temperature-sensitive lambda-repressor cItS857 gene, lambda PR and PL promoters was amplified by polymerase chain reaction (PCR) from pND707 using the following primers: pND_fw_PstI (CTGCAGGTGATGAT TATCAGCCAGCAG) and pND_rv_BamHI (GGATCCC AATGCTTCGTTTCGTATCAC). After gel purification and digestion with PstI and BamHI, the PCR product was cloned into pACYC177, resulting in the plasmid of pSV. Plasmids pSV01, pSV02, and pSV03 were obtained by inserting different genes into plasmid pSV (Table 1).

Isolation, manipulation, and transformation of DNA

Plasmid DNA was isolated using the Plasmid Mini-Preps Kit (BIO Basic Inc.). Chromosomal DNA from *E. coli* W3110 was prepared by using the Genomic DNA Isolation Kit (BIO Basic Inc.). Agarose gel purification of DNA fragments was performed using the Takara Agarose Gel

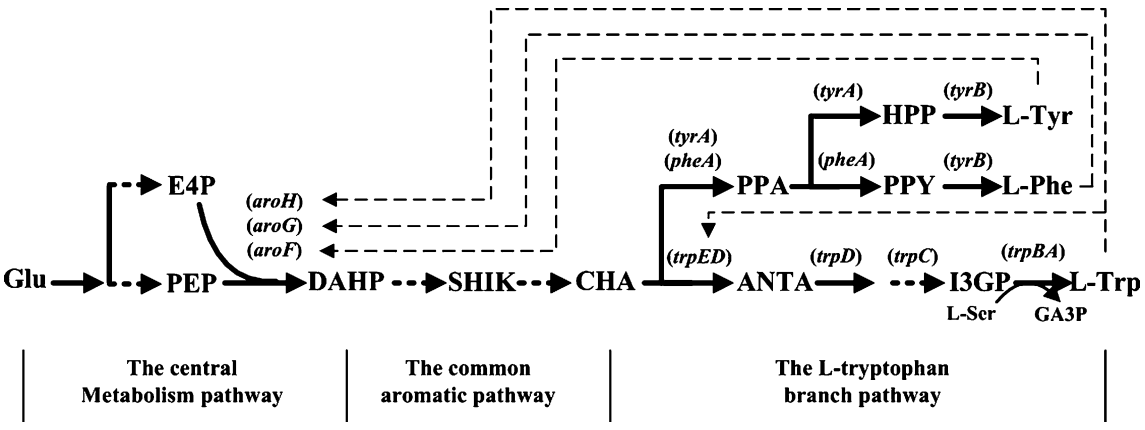

Fig. 1 L-Trp biosynthesis and its regulations in *E. coli*. The *dashed lines* indicate transcriptional and allosteric control exerted by the aromatic amino acid end products. Abbreviations used: *ANTA* anthranilate, *CHA* chorismate, *DAHP* 3-deoxy-D-arobino-heptulosonate 7-phosphate, *E4P* erythrose 4-phosphate, *HPP* 4-hydroxyphenlypyruvate, *I3GP* indole 3-glycerolphosphate, *L-Phe* L-phenylalanine, *L-Trp* L-tryptophan, *L-Tyr* L-tyrosine, *PEP* phosphoenolpyruvate, *PPA* prephenate, *PPY* phenylpyruvate, *SHIK* shikimate

Table 1 Strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
W3110	K-12 wild-type	ATCC 27325
FB-01	W3110(Δ*trpR*)	This work
FB-02	W3110(Δ*trpR*, Δ*tnaA*)	This work
FB-03	W3110(Δ*trpR*, Δ*tnaA*, Δ*pheA*)	This work
FB-04	W3110(Δ*trpR*, Δ*tnaA*, Δ*pheA*, Δ*tyrA*)	This work
Plasmid		
pACYC177	Low copy number, p15A replicon, amp and kan markers	CICIM
pND707	Temperature-sensitive lambda-repressor, Lambda PR and PL promoter	CICIM
pKD13	amp and kan markers	[3]
pKD46	amp marker, helper plasmid	[3]
pCP20	amp and chl markers, helper plasmid	[3]
pSV	Based on plasmids of pACYC177 and pND707, p15A replicon, kan markers, lambda PR and PL promoter	This work
pSV01	pSV derivative, carrying $aroF^{wt}$	This work
pSV02	pSV derivative, carrying $aroF^{fbr}$	This work
pSV03	pSV derivative, carrying $aroF^{fbr}$ and $trpE^{fbr}D$	This work

DNA Purification Kit Ver.2.0 (Takara Biotechnology Co. Ltd). Restriction enzymes, ligases, and other DNA-manipulating enzymes were used according to the manufacturer's manual. All plasmid constructs were verified by DNA sequencing (Bio Basic Inc.). Plasmid DNA was transferred to competent cells of *E. coli* by electroporation.

Cloning and expression of $aroF^{fbr}$ and $trpE^{fbr}D$

The wild-type *aroF* ($aroF^{wt}$) and *trpED* ($trpED^{wt}$) were amplified by PCR from genomic DNA of *E. coli* W3110 using the following primers: aroF_fw_BglII (AGATC TATGCAAAAAGACGCGCTG), aroF_rv_BglII (AGAT CTTTAAGCCACGCGAGCCGTC) and trpED_fw_BamHI (GGATCCATGCAAACACAAAAACCGACTC), trpED_rv_BamHI (GGATCCTTACCCTCGTGCCGCCAGTG), respectively. After gel purification, $aroF^{wt}$ and $trpED^{wt}$ were ligated into pMD18-T Vector (Takara Biotechnology Co. Ltd). The plasmids pMD18/$aroF^{wt}$ and pMD18/$trpED^{wt}$ were transformed into *E. coli* JM109, respectively.

The feedback inhibition-resistant AroF ($AroF^{fbr}$), which had an Ile11 deletion of AroF, was obtained by site-directed mutagenesis using plasmid pMD18/$aroF^{wt}$ as template and a pair of complementary primers aroF_mfw (CGCTGAATAACGTACATACCGACGAACAG) and aroF_mrv (CCTGTTCGTCGGTATGTACGTTATTCAG). The PCR product was treated with DpnI and transformed into *E. coli* JM109. The feedback inhibition-resistant TrpED ($TrpE^{fbr}D$), which had Ser40Phe mutation of TrpE [6], was obtained by site-directed mutagenesis using pMD18/$trpED^{wt}$ as template and a pair of complementary primers trpED_mfw (CTGGAATTCGCAGATATCGAC AGCAAAG) and trpED_mrv (GCTGTCGATATCTGC GAATTCCAG). All the mutations were verified by DNA sequencing.

The $aroF^{wt}$ and $aroF^{fbr}$ were subcloned into plasmid pSV, respectively, under the control of the PR promoter, to create plasmids pSV01and pSV02. The $trpE^{fbr}D$ was subcloned into pSV02 under the control of the PL promoter, resulting in pSV03.

Construction of gene knock-out mutants

Single- or multi-gene knock-out mutants were constructed by one-step inactivation method reported by Baba et al. [3] using the primers in Table 2. In these mutant strains, the target gene sequence in addition to its initiation codon, C-terminal 18-nt coding region, and stop codon was replaced with a kanamycin (kan) resistance gene. After selection, the kan resistance gene was eliminated by using a helper plasmid [3]. All gene knock-out strains were verified by sequencing.

Media and cultivation

In a shake flask, *E. coli* W3110 and its derivatives were incubated in Luria–Bertani (LB) medium which contained 10 g/l peptone; 10 g/l NaCl; 5 g/l yeast extract. The fermentation medium used in a 3-l fermentor was modified

Table 2 Oligonucleotide primers for construction of the gene knock-out strains

Gene	Name	Primers 5′ → 3′
trpR	trpR_fw	AACCGGGGGAGGCATTTTGCTTCCCCCGCTAACAATGGCGACATATTATGATTCCGGGGATCCGTCGACC
	trpR_rv	ATGCGCCACGTCTTATCAGGCCTACAAAATCAATCGCTTTTCAGCAACACTGTAGGCTGGAGCTGCTTCG
tnaA	tnaA_fw	ACATCCTTATAGCCACTCTGTAGTATTAATTAAACTTCTTTAAGTTTTGCATTCCGGGGATCCGTCGACC
	tnaA_rv	AATATTCACAGGGATCACTGTAATTAAAATAAATGAAGGATTATGTAATGTGTAGGCTGGAGCTGCTTCG
pheA	pheA_fw	CTCCCAAATCGGGGGGCCTTTTTTATTGATAACAAAAAGGCAACACTATGATTCCGGGGATCCGTCGACC
	pheA_rv	GATGATTCACATCATCCGGCACCTTTTCATCAGGTTGGATCAACAGGCACTGTAGGCTGGAGCTGCTTCG
tyrA	tyrA_fw	GATGATGTGAATCATCCGGCACTGGATTATTACTGGCGATTGTCATTCGCATTCCGGGGATCCGTCGACC
	tyrA_rv	GGATCTGAACGGGCAGCTGACGGCTCGCGTGGCTTAAGAGGTTTATTATGTGTAGGCTGGAGCTGCTTCG

The *underlines* indicate 50-nt homology extensions of a target knock-out gene

according to Gerigk et al. [10], comprising 3 g/l $MgSO_4{\cdot}7H_2O$, 0.015 g/l $CaCl_2{\cdot}H_2O$, 3 g/l KH_2PO_4, 1 g/l NaCl, 5 g/l $(NH4)_2SO_4$, 0.07 g/l $FeSO_4{\cdot}7H_2O$, 0.1 g/l Na-Citrate, 0.2 g/l Yeast Extract, 8 g/l glucose, and 1.5 ml/l trace element solution (2 g/l $Al_2(SO_4)_3{\cdot}18H_2O$, 0.75 g/l $CoSO_4{\cdot}7H_2O$, 2.5 g/l $CuSO_4{\cdot}5H_2O$, 0.5 g/l H_3BO_3, 2.4 g/l $MnSO_4{\cdot}H_2O$, 3 g/l $Na_2MoO_4{\cdot}2H_2O$, 2.5 g/l $NiSO_4{\cdot}6H_2O$, 15 g/l $ZnSO_4{\cdot}7H_2O$). When fed-batch fermentation of strain FB-04/pSV03 was performed, 2 g/l L-Phe and 3 g/l L-Tyr were supplemented to the medium. Antibiotics were used when needed at the following final concentration: 40 μg/ml of kan and 100 μg/ml of ampicillin (amp). Feeding solution contained 500 g/l glucose. The pH value was adjusted to 6.9 with 2 M NaOH.

A stirred 3-l glass vessel with the BioFlo110 modular fermentor system (New Brunswick Scientific, Edison, NJ, USA) was used. The inoculum ratio was 10% (v/v). When glucose in the medium was nearly exhausted, feeding solution was started. The feeding rate of glucose was flexible so that the concentration of glucose in the broth did not exceed 5 g/l. The culture temperature of 33°C was stepped up to 38°C when the cells were grown up to the mid-exponential phase of growth. The pH was controlled at 6.8 with NH_4OH. The dissolved oxygen (DO) concentration was kept at 30% via changing fermentor agitation speed and aeration rate.

Protein quantification and SDS-PAGE analysis

Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad), with purified bovine serum albumin (Promega) as the standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% acrylamide gels and the proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Enzymatic activity determinations

The DAHP synthase activity was determined according to the method described previously [25]. One unit of enzyme activity is defined as the production of 1 μmol of DAHP or the disappearance of 1 μmol of PEP per minute. The ANTA synthase activity was determined according to the method of Zalkin and Kling [32]. One unit of enzyme activity is defined as the production of 1 nmol of ANTA per minute.

Analysis of fermentation parameters

The concentration of three aromatic acids and ANTA in the broth was determined by RP-HPLC on an Agilent 1200 HPLC system with an Inertsil ODS-SP column (250 mm × 4.6 mm i.d., 5 μm, GL Sciences, Japan). A mixture solution of methanol (A) and 0.05% H_3PO_4 (B) as mobile phase was used for gradient elution (0–3 min, 2% A; 3–22 min, 2–80% A, and maintained to 25 min; 25–26 min, 80–2% A, returned to initial condition) at a flow rate of 0.8 ml/min. UV absorbance of the effluent was monitored at 210 nm. Glucose was monitored during fermentation using glucose-glutamate analyzer SBA-40C (Biology Institute of Shandong Academy of Sciences, Jinan, China). The cell concentration was determined from optical density (OD 600) and the cell dry weight (CDW) was determined by a pre-calibrated relationship (1 OD = 0.492 g/l CDW).

Results and discussion

The development of the strain W3110/pSV-*aroF*^{fbr}-*trpE*^{fbr}*D*

As a prerequisite for L-Trp production, the enzymes involved in feedback inhibition of L-Trp biosynthesis have to be deregulated. In *E. coli*, these enzymes mainly include DAHP synthase in the common aromatic pathway and ANTA synthase in the L-Trp branch pathway. In previous literature, the removal of feedback inhibition of the DAHP synthase was often considered at first [5, 29].

In *E. coli*, there exist three kinds of DAHP synthase isoenzymes, AroF, AroG, and AroH, which are subject to feedback inhibition by L-Tyr, L-Phe, and L-Trp, respectively. In wild-type cells growing in minimal medium, the AroG and AroF isoenzymes make up most of the total DAHP synthase activity, and the AroH isoenzyme makes up only about 1% [27], thus in the strains of aromatic amino acids production, feedback inhibition of DAHP synthases is usually overcome by expression of the feedback-resistant AroF or AroG isoenzyme [16, 17, 28]. In the present study, the removal of feedback inhibition of AroF isoenzyme was performed.

Currently, there is no report of crystal structure of AroF isoenzyme, however, a high degree of similarity in sequence and kinetic characteristics of the three DAHP synthase isoenzymes in *E. coli* strongly suggests that they have similar three-dimensional structure and inhibition mechanism [16]. Sequence alignment showed that Ile10 of AroG and Ile11 of AroF are conserved residues. Furthermore, from crystal structure of AroG, the inhibitor binding site is in a cavity located on the outer side of the N-terminal end of $(\beta/\alpha)_8$ barrel structure and Ile10 is one of the critical residues surrounding the inhibitor [26]. Previously, it has been reported that the replacement of residue Ile10 of AroG with Ala caused a 70% loss of enzyme activity, even though the feedback inhibition of enzyme was desensitized [12]. In the present study, in order to achieve both deregulated and active AroF, it was attempted to delete Ile11 residue by site-directed mutagenesis. The mutated AroF was named as $AroF^{fbr}$.

Both $aroF^{wt}$ and $aroF^{fbr}$ were cloned into the expression vector pSV next to the PR promoter, resulting in the plasmids pSV01 and pSV02, and the recombinant plasmids were transformed into W3110, respectively. The specific activities of DAHP synthase in crude extracts of *E. coli* W3110/pSV01 and W3110/pSV02 were determined to be 13.2 U/mg and 12.9 U/mg, respectively. Since SDS-PAGE analysis showed that the enzyme expression level in both strains were similar (data not shown), it is considered that the mutation did not cause the decrease of the enzyme activity. In order to investigate the feedback resistance of the enzyme, the activities of DAHP synthase in the crude extract of both strains were assayed in the presence of inhibitor L-Tyr. The results showed that DAHP synthase activity of *E. coil* W3110/pSV01 was inhibited by 40% at 0.03 mM of L-Tyr and retained less than 5% residual enzyme activity at 0.5 mM of L-Tyr. In contrast, DAHP synthase activity of *E. coli* W3110/pSV02 was not inhibited even in the presence of 5 mM of L-Tyr (Fig. 2). In order to determine whether a high level of L-Trp inhibits the activity of $AroF^{fbr}$, 5 g/l of L-Trp was added into the enzyme assay solution of crude extract of *E. coli* W3110/pSV02. The results showed that the DAHP synthase activity was fully retained.

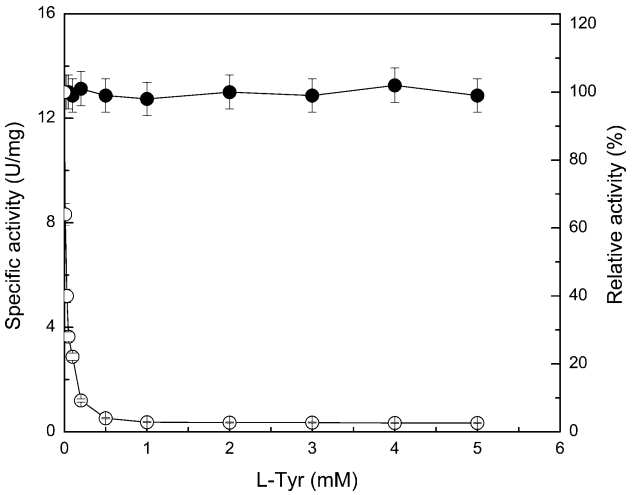

Fig. 2 L-Tyr inhibition patterns of DAHP synthases. $AroF^{wt}$ (*open circle*), $AroF^{fbr}$ (*filled circle*). The data represent the means ± SD from three measurements

ANTA synthase encoded by *trpED* is another key enzyme that is feedback-regulated by L-Trp (Fig. 1). Previously, it has been reported that the mutated TrpE(S40F)D was able to relieve the feedback regulation by L-Trp and exhibited similar specific ANTA synthase activity compared to $TrpED^{wt}$ [6, 31]. In the present study, the encoding gene of TrpE(S40F)D, named $trpE^{fbr}D$, was cloned into plasmid pSV02 next to the PL promoter resulting in the plasmid pSV03. The specific activity of TrpE(S40F)D in crude extracts of *E. coli* W3110/pSV03 was determined to be 54.2 U/mg, which was similar to that of $TrpED^{wt}$ (53.6 U/mg). Since SDS-PAGE analysis showed that the enzyme expression level in both strains was similar (data not shown), it is considered that the mutation did not cause the decrease of the enzyme activity.

To evaluate the L-Trp production capability of *E. coli* W3110-harboring plasmids of pSV, pSV01, pSV02, or pSV03, respectively, fed-batch fermentation was performed in a 3-l fermentor. The results showed that W3110/pSV did not excrete L-Trp in detectable amounts and W3110/pSV01 only produced 0.05 g/l of L-Trp after 30 h of incubation; W3110/pSV02 produced 0.23 g/l of L-Trp, which was 3.6-fold higher when compared to that of W3110/pSV01; while W3110/pSV03 produced 0.87 g/l of L-Trp, which was 16.4-fold higher when compared to that of W3110/pSV01. In addition, it was noted that two competitive amino acids, L-Phe and L-Tyr, and a precursor of L-Trp, ANTA, were accumulated in the culture medium together with the increase of concentration of L-Trp. The strain W3110/pSV03 can produce 0.41 g/l L-Phe, 0.27 g/l L-Tyr, and 0.58 g/l ANTA after 30 h of incubation.

The development of *trpR* knock-out mutant

In addition to feedback regulation, there still exist some other regulatory mechanisms and competing pathways during the biological synthesis of L-Trp in *E. coli*. For example, the product of *trpR* in *E. coli* represses the transcription of genes involved in L-Trp synthesis and transport [11]. In the present study, *trpR* gene of W3110/pSV03 was knocked out by using the Red recombination system and the *trpR* knock-out strain was named as FB-01/pSV03. Fermentation results showed that the production of L-Trp of FB-01/pSV03 was increased to 2.8 g/l, which is 3.2-fold higher when compared to that of the control strain W3110/pSV03 (Fig. 3a; Table 3). In addition, the concentrations of L-Phe, L-Tyr, and ANTA in the culture medium were increased to 1.8, 1.4, and 2.3 g/l, respectively (Fig. 3a).

The development of *tnaA* knock-out mutant

As reported previously, the tryptophanase encoded by *tnaA* catalyzes the degradation of L-Trp to indole, pyruvate, and ammonia. The removal of the *tnaA* gene can greatly improve the production of L-Trp [1, 22]. In the present study, the *tnaA* gene of FB-01/pSV03 was further knocked out based on Δ*trpR* background and resulted in the strain of FB-02/pSV03. Strain FB-02/pSV03 produced 7.8 g/l L-Trp, which is 9.0-fold and 2.7-fold higher when compared to that of the strain W3110/pSV03 and FB-01/pSV03, respectively (Fig. 3b; Table 3). However, more byproducts were accumulated in the culture and the concentrations of L-Phe, L-Tyr, and ANTA reached 5.3, 3.6, and 5.4 g/l, respectively (Fig. 3b).

The development of *pheA* knock-out mutant

The L-Trp production capacity of FB-02/pSV03 was significantly increased by knocking out both *trpR* and *tnaA* genes, but from the profile of accumulation of L-Phe and L-Tyr in Fig. 3b, more and more carbon flux was redirected from CHA to the L-Phe and L-Tyr branch pathways and L-Trp productivity decreased dramatically to zero during the latter stage of fermentation. Such decrease of L-Trp productivity has been reported previously and it was suggested to be relevant to the enzyme feedback regulation [2, 14]. Although the AroF and TrpED had been deregulated with the increase of intracellular concentration of L-Trp during fermentation progress, it may inhibit other important regulatory enzymes such as tryptophan synthase, which can lead to the L-Trp carbon flux blockage and thus aberrantly shifting that carbon flux to L-Phe and L-Tyr pathways (Fig. 1).

In order to prevent the above-mentioned carbon loss, in the present study, L-Phe and L-Tyr synthesis pathways were attempted to be blocked. Based on Δ*trpR*, Δ*tnaA* background, *pheA*, which encodes a bifunctional enzyme catalyzing the first two reaction steps of the L-Phe branch

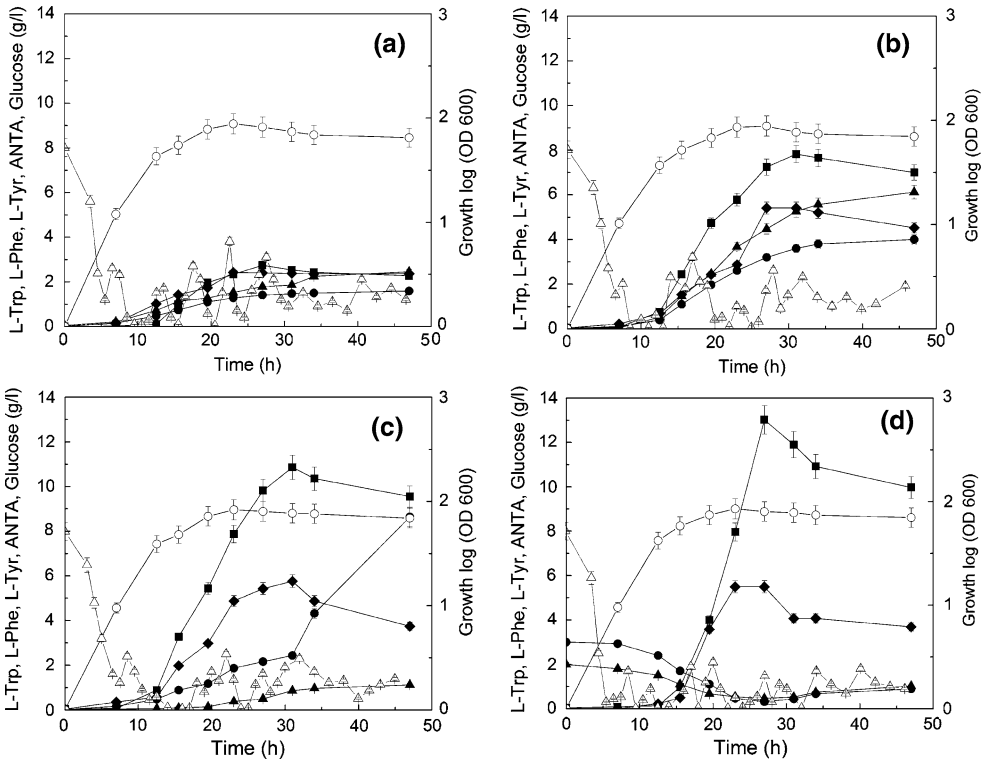

Fig. 3 Fed-batch fermentations of *E. coli* FB-01/pSV03 (**a**), FB-02/pSV03 (**b**), FB-03/pSV03 (**c**), and FB-04/pSV03 (**d**). Cell growth (*open circle*), L-Trp concentration (*filled square*), L-Phe concentration (*filled triangle*), L-Tyr concentration (*filled circle*), ANTA concentration (*filled diamond*) and glucose concentration (*open triangle*). The data represent the means ± SD from three measurements

Table 3 Comparison of fermentation parameters of different gene knock-out strains

Parameter	FB-01/pSV03	FB-02/pSV03	FB-03/pSV03	FB-04/pSV03
Max. cell dry weight (CDW; g/l)	43.2 ± 2.0	42.1 ± 2.5	40.8 ± 2.3	41.2 ± 2.5
Glucose consumption (g/l)	142 ± 10	158 ± 12	146 ± 8	138 ± 6
Average specific growth rate μ (h^{-1})	0.11 ± 0.006	0.12 ± 0.003	0.13 ± 0.003	0.13 ± 0.002
Max. L-Trp titer (g/l)	2.8 ± 0.3	7.8 ± 0.5	10.8 ± 1.0	13.3 ± 0.9
Max. productivity ($g_{Trp}/g_{CDW}*h^{-1}$)	0.022 ± 0.001	0.022 ± 0.002	0.028 ± 0.005	0.029 ± 0.007
Conversion ratio ($g_{Trp}/g_{glucose}$)	0.02 ± 0.001	0.05 ± 0.002	0.07 ± 0.002	0.10 ± 0.005

The data represent the means ± SD from three measurements

pathway in *E. coli*, was further knocked out and the resulting strain was named FB-03/pSV03. The fermentation results showed that the L-Trp production in the culture media of FB-03/pSV03 was increased to 10.8 g/l, which is 1.38-fold higher when compared to that of FB-02/pSV03 (Fig. 3c; Table 3). In addition, the concentration of L-Tyr in the culture medium was increased to 5.1 g/l and the concentration of ANTA did not change (Fig. 3c). These results showed that the carbon flux of both L-Trp and L-Tyr branch pathways increased under the blockage of L-Phe branch pathway.

In addition, a small amount of L-Phe (0.9 g/l) was observed in the culture medium of FB-03/pSV03 after cultivation of 30 h. This phenomenon could be due to the fact that in *E. coli* cells, besides PheA, another bifunctional enzyme, chorismate mutase/prephenate dehydrogenase (encoded by *tyrA*), can also catalyze the first catalytic step of L-Phe branch pathway (Fig. 1). So, in the strain of knocking out *pheA* alone, CHA was also able to be converted into prephenate by TyrA. Furthermore, it has been reported that prephenate could be non-enzymatically converted to phenylpyruvate [30, 33], which could eventually be transaminated to L-Phe.

The development of *tyrA* knock-out mutant

Based on Δ*trpR*, Δ*tnaA*, and Δ*pheA* background, *tyrA* was further knocked out on genome and resulted in FB-04/pSV03. When *pheA* and *tyrA* were both knocked out, CHA could not be converted into prephenate, and as a result there was no synthesis of L-Phe or L-Tyr. So only by adding the appropriate amount of L-Phe and L-Tyr into the medium could FB-04/pSV03 grow normally and the appropriate concentration was determined to be 2 g/l for L-Phe and 3 g/l for L-Tyr. According to our understanding, the reason for the large amount of L-Phe and L-Tyr required is not yet clear, but may be that these two amino acids, besides their role in cell growth, also participate in other unknown metabolic activities in the cell. As for the L-Trp production, the results of the batch fermentation of FB-04/pSV03 indicated that it was further improved to 13.3 g/l, 15.2-fold when compared to that of the control strain W3110/pSV03, and no obvious change in the concentration of ANTA was observed (Fig. 3d; Table 3).

In order to compare the L-Trp production of strains (FB-01, 02, 03, and 04) with or without plasmids of pSV01 or pSV02, the fed-batch fermentation of these strains was performed. The results showed that the strains (FB-01, 02, 03, and 04) without any extra plasmid or harboring pSV01 produced similar amounts of L-Trp, which were all less than 0.3 g/l, when these strains harboring pSV02, they produced less than 0.6 g/l L-Trp. In addition, the L-Trp productivities of all of the above strains were less than $0.01 g_{Trp}/g_{CDW}*h^{-1}$ and no detectable L-Phe, L-Tyr, and ANTA were observed in these culture media. These results, compared to that from the strains (FB-01, 02, 03, and 04) harboring pSV03, indicated that L-Trp production could be significantly improved when feedback inhibitions of both DAHP synthase and ANTA synthase were eliminated.

Our results showed a set of defined genetic manipulations in *E. coli* which greatly increased the production of L-Trp. Although the L-Trp production of FB-04/pSV03 (13.3 g/l) was lower than that of the strains constructed by Azuma et al. (54.6 g/l) and Dodge et al. (42 g/l) [2, 9] and the conversion ratio from glucose to L-Trp of FB-04/pSV03 (10%) was also lower than that of the strain constructed by Dodge et al. (18%) [2, 9], FB-04/pSV03 has completely defined genome traits. A lot of new explorations on biosynthetic mechanism of L-Trp [4, 19, 23] and various large-scale analytical techniques such as transcriptome and proteome analysis [20, 24], which are difficult to be applied on the random mutagenesis strains because of unknown mutations in their genomes, can provide new information for the further improvement of the strain FB-04/pSV03.

Conclusions

In the present study, the feedback-resistant AroF was achieved. An L-Trp production strain FB-04/pSV03 was developed by expressing the $AroF^{fbr}$ and $TrpE^{fbr}D$ in the

multi-gene (*trpR*, *tnaA*, *pheA*, and *tyrA*) knock-out strain. The constructed strain FB-04/pSV03 produced 13.3 g/l L-Trp with 10% conversion ratio from glucose, while there was no detectable L-Trp in its parent strain. Further strain development would be achieved through genetic optimization and fermentation process development. In addition, the construction of L-Trp production strain by genetically defined modification can also be used to help understand the synthetic mechanism of L-Trp through metabolic pathway analysis.

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