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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Enhanced production of L-phenylalanine in *Corynebacterium glutamicum* due to the introduction of *Escherichia coli* wild-type gene *aroH*

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Abstract Metabolic engineering is a powerful tool which has been widely used for producing valuable products. For improving L-phenylalanine (L-Phe) accumulation in *Corynebacterium glutamicum*, we have investigated the target genes involved in the biosynthetic pathways. The genes involved in the biosynthesis of L-Phe were found to be strictly regulated genes by feedback inhibition. As a result, overexpression of the native wild-type genes *aroF*, *aroG* or *pheA* resulted in a slight increase of L-Phe. In contrast, overexpression of $aroF^{wt}$ or $pheA^{fbr}$ from *E. coli* significantly increased L-Phe production. Co-overexpression of $aroF^{wt}$ and $pheA^{fbr}$ improved the titer of L-Phe to 4.46 ± 0.06 g l^{-1}. To further analyze the target enzymes in the aromatic amino acid synthesis pathway between *C. glutamicum* and *E. coli*, the wild-type gene *aroH* from *E. coli* was overexpressed and evaluated in *C. glutamicum*. As predicted, upregulation of the wild-type gene *aroH* resulted in a remarkable increase of L-Phe production. Co-overexpression of the mutated $pheA^{fbr}$ and the wild-type gene *aroH* resulted in the production of L-Phe up to 4.64 ± 0.09 g l^{-1}. Based on these results we conclude that the wild-type gene *aroH* from *E. coli* is an appropriate target gene for pathway engineering in *C. glutamicum* for the production of aromatic amino acids.

Keywords L-phenylalanine · *Corynebacterium glutamicum* · *aroH* · Metabolic engineering

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Introduction

L-phenylalanine (L-Phe), one of the essential amino acids for humans and other animals, is widely used in food and pharmaceutical industries [1, 29, 33]. In early industrial processes L-Phe was mainly produced by chemical synthesis. However, because of the very specific demand for the stereo-specific form, consumer's preference, and various problems with chemical synthesis, the latter is gradually being replaced with bioprocessing, such as microbial fermentation and enzymatic transformation [22]. More recently, metabolic engineering has focused on the Gram-negative strain *Escherichia coli* and many other well-characterized model microorganisms, and cell factories have been constructed [13, 24, 33]. Although high titers of L-Phe can be produced, the industrial-scale production of L-Phe by genetically engineered *E. coli* has encountered a lot of problems, especially infection by phage. This has led to the search for alternative robust engineered strains (e.g. *Corynebacterium glutamicum*).

Since 1957, *C. glutamicum*, a well-known Gram-positive strain in industrial processes, has been widely used for

producing amino acids, especially L-glutamate and L-lysine [9, 26]. The whole genome sequencing of *C. glutamicum* has led to the successful development of many sophisticated vectors which have been applied in *C. glutamicum* [6, 18, 23, 30]. In recent years, researchers have taken advantage of the powerful toolbox that has become available ("omics" technologies, metabolic pathway engineering, targeted enzyme modification) and successfully engineered *C. glutamicum* for producing many high valuable-added products, as described in the excellent review by Becker and Wittmann [2]. Due to the attractive features of genetically engineered *C. glutamicum*, such as fast growth rate, lack of endotoxin and protease secretion and easy purification step [6], *C. glutamicum* has also been used for the heterologous expression of recombinant proteins [3, 4, 20]. In addition, *C. glutamicum* has a distinct ability to simultaneously metabolize pentose and hexose, which makes this microorganism a potential candidate for transforming renewable biomasses to value-added products [9].

C. glutamicum's L-Phe biosynthesis pathway, similar to that *E. coli*, is divided into three parts: central carbon metabolism, the shikimate pathway and the chorismate pathway [15]. Much research has focused on elucidating this pathway in *E. coli*, and the enzyme 3-deoxy-7-phosphoheptulonate synthase (DS, encoded by *aroG*, *aroF* and *aroH*) and the bifunctional enzyme chorismate mutase-*p*-prephenate dehydratase (CM-PDT, encoded by *pheA*) have been identified as the committed enzymes [7, 14, 25]. In *E. coli*, the three DS isozymes encoded by *aroG*, *aroF* and *aroH* are feedback inhibited by L-Phe, L-tyrosine (L-Tyr) and L-tryptophan (L-Trp), respectively (Fig. 1a) [10, 13, 27]. In *C. glutamicum*, the two DS isozymes, which are encoded by *aroG* and *aroF*, are feedback inhibited by L-Phe, chorismate and prephenate and by L-Tyr and L-Tyr, respectively (Fig. 1b) [6, 21, 27]. Nevertheless, in *E. coli*, the bifunctional enzyme CM-PDT is feedback inhibited by L-Phe and L-Tyr. In *C. glutamicum*, the enzymes CM and prephenate dehydratase (PD) are encoded by *csm* [21] and *pheA* [16], respectively. Further studies have demonstrated that CM activity is not affected by L-Phe, L-Tyr or L-Trp [21] while PD activity is feedback inhibited by L-Phe [6, 12].

To accumulate L-Phe efficiently in *E. coli*, the *aroG* and *pheA* genes from *E. coli* were modified by site-mutation to eliminate the specific feedback inhibition and overexpressed to increase the production of L-Phe [1, 8, 31, 33]. In a similar approach, the mutated *pheA* gene of *E. coli* was introduced into *C. glutamicum* KY10694, and a 30 % increase of L-Phe was achieved [17]. In one study, integration of the *aroG-pheA* tandem genes of *E. coli* into *C. glutamicum* resulted in a 1.71-fold increase in L-Phe production, although the highest concentration was also very low (3.97 g l^{-1}) in shake flask cultivation [22]. Shu et al. [24] achieved a high level of L-Phe

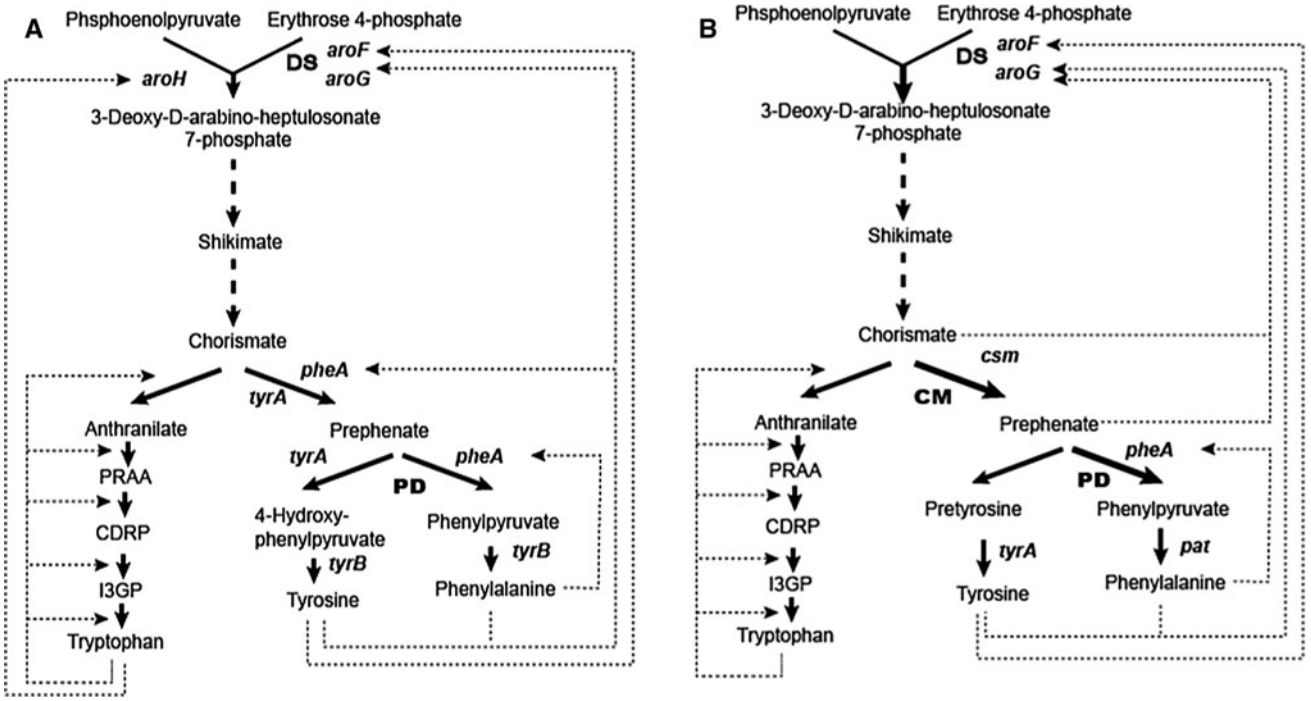

Fig. 1 L-phenylalanine (L-Phe) biosynthesis pathway in *Escherichia coli* (**a**) and *Corynebacterium glutamicum* (**b**). *Dotted lines*, *dashed lines* feedback inhibition and repression, respectively. *DS* 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase, *CM* chorismate mutase, *PD* prephenate dehydratase, *CDRP* 1-(2-carboxyphenylamino)-1-deoxy-D-nbulose-5-phosphate, *I3GP* indole 3-glycerolphosphate, *PRAA* *n*-(5-phospho-β-D-nbosylanthranilate), *PRT* anthranilate phosphoribosyltransferase

(23.2 g l^{-1}) by supplying elevated oxygen into 5-l fermentor with the wild-type strain. In a previous study in *E. coli* in which we analyzed the key enzymes of the shikimate and chorismate pathways in *C. glutamicum* ATCC 13032, we found that the wild-type gene *aroH* that is inhibited by L-Trp from *E. coli* was expressed in *C. glutamicum* and contributed significantly to increased L-Phe production. By co-overexpression of mutated *pheA* and the wild-type *aroH* gene, L-Phe was increased by 13.6-fold. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reverse transcription (RT) PCR data showed that the levels of the mutated *pheA* and wild-type *aroH* within one operon were significantly different, suggesting that precise regulation of the target enzymes might be beneficial to the titer of the target end-product, L-Phe.

Materials and methods

Bacterial strains and plasmids

Bacterial strains, plasmids and oligonucleotides used in this study are listed in Tables 1 and 2. *E. coli* JM109 was applied for molecular cloning and manipulation of plasmids. *C. glutamicum* ATCC 13032 as the parent strain was engineered for producing L-Phe. Genes *aroG*, *aroF* and *pheA* were amplified from genomic DNA of *C. glutamicum* ATCC 13032 while the wild-type gene *aroH* was amplified from genomic DNA of *E. coli* W3110. After digestion, the fragments were subcloned into pXMJ19 to generate pXMJ19-*aroG*, pXMJ19-*aroF*, pXMJ19-*pheA* and pXMJ19-*aroH*, respectively. *PheA*^{fbr} and *aroF*^{wt} were amplified from plasmid pAP-B03 [33] and subcloned into pXMJ19, resulting in plasmids pXMJ19-*pheA*^{fbr} and pXMJ19-*aroF*^{wt}, respectively. To increase the translation efficiency, the Shine-Dalgarno sequence (AGGAGGA) was artificially added in the upstream of starting codon ATG (Table 1).

Corynebacterium glutamicum transformation

Corynebacterium glutamicum competent cells for electroporation were prepared by the method described by Xu et al. [30]. Briefly, a colony of *C. glutamicum* was inoculated into 30 ml of LBG (LB medium with 5 g l^{-1} glucose) and cultivated overnight for about 16 h at 30 °C

Table 1 Strains and plasmids used in this study

Strains and plasmids	Relevant properties	Source or reference
Strains		
Escherichia coli JM109	Wide-type *E. coli.*	Lab stock
E. coli W3110	In(rrnD-rrnE) *E. coli*	Lab stock
Corynebacterium glutamicum ATCC 13032	Wide-type *C. glutamicum*	ATCC
C. glutamicum 19G	*C. glutamicum* ATCC 13032 harboring pXMJ19- *aroG*	This work
C. glutamicum 19F	*C. glutamicum* ATCC 13032 harboring pXMJ19- *aroF*	This work
C. glutamicum 19F^{wt}	*C. glutamicum* ATCC 13032 harboring pXMJ19- *aroF*^{wt}	This work
C. glutamicum 19H	*C. glutamicum* ATCC 13032 harboring pXMJ19- *aroH*	This work
C. glutamicum 19A	*C. glutamicum* ATCC 13032 harboring pXMJ19-*pheA*	This work
C. glutamicum 19A^{fbr}	*C. glutamicum* ATCC 13032 harboring pXMJ19-*pheA*^{fbr}	This work
C. glutamicum 19A^{fbr}H	*C. glutamicum* ATCC 13032 harboring pXMJ19-*pheA*^{fbr}-*aroH*	This work
C. glutamicum 19A^{fbr}F^{wt}	*C. glutamicum* ATCC 13032 harboring pXMJ19-*pheA*^{fbr}-*aroF*^{wt}	This work
Plasmids		
pMD19-simple Vector	Cloning vector, Amp^{R}	Takara, Dalian, China
pXMJ19	*E. coli-C. glutamicum* shuttle expression vector	[6]
pAP-B03	Derivative from pACYC177 and pPL450, containing *pheA*^{fbr} and *aroF*^{wt}	[33]
pXMJ19-*aroG*	pXMJ19 containing *aroG* (*C. glutamicum* ATCC 13032)	This work
pXMJ19-*aroF*	pXMJ19 containing *aroF* gene (*C. glutamicum* ATCC 13032)	This work
pXMJ19-*aroF*^{wt}	pXMJ19 containing *aroF*^{wt} gene (pAP-B03)	This work
pXMJ19-*aroH*	pXMJ19 containing *aroH* (*E. coli* 3110)	This work
pXMJ19-*pheA*	pXMJ19 containing *pheA* (*C. glutamicum* ATCC 13032)	This work
pXMJ19-*pheA*^{fbr}	pXMJ19 containing *pheA*^{fbr} (pAP-B03)	This work
pXMJ19-*pheA*^{fbr}-*aroH*	pXMJ19 containing *pheA*^{fbr} (pAP-B03) and *aroH* (*E. coli*)	This work
pXMJ19-*pheA*^{fbr}-*aroF*^{wt}	pXMJ19 containing *pheA*^{fbr} (pAP-B03) and *aroF*^{wt} (pAP-B03)	This work

ATCC American type culture collection

Table 2 Primers used in this study

Primers and reverse transcription primers	Sequence[a]
Primers	
aroG-F	5′-CTAGTCTAGAAA**AGGAGG**ACACGCATGAGTTCTCCAGTCTCACTCGAAAA-3′
aroG-R	5′-TCCCCCGGGTTACTTGGCTGCTGCTCGGC-3′
aroF-F	5′-CCCAAGCTTAA**AGGAGG**ACACGCATGAGTTCTCCAGTCTCACTCGAAAA-3′
aroF-R	5′-CGCGGATCCTTACTTGGCTGCTGCTCGGC-3′
aroF^{wt}-F	5′-TCCCCGCGGAAA**AGGAGG**ACACGCATGCAAAAAGACGCGCTGAATAACG-3′
aroF^{wt}-R	5′-CGCGGATCCCCGCTCGAGTTAAGCCACGCGAGCCGTCA-3′
aroH-F	5′-CGCGGATCCAA**AGGAGG**ACACGCATGAACAGAACTGACGAACTCCGTAC-3′
aroH-R	5′-TCCCCCGGGCCGCTCGAGTCAGAAGCGGGTATCTACCGCA-3′
pheA-F	5′-CCCAAGCTTAA**AGGAGG**ACACGC ATGAGCGACGCACCAACTGTTG-3′
pheA-R	5′-TCCCCCGGG CTAGTTAAGTTTCCTTCCTTCGCTTGCT-3′
pheA^{fbr}-F	5′-CCCAAGCTTAA**AGGAGG**ACACGCATGACATCGGAAAACCCGTTACT-3′
pheA^{fbr}-R	5′-TGCACTGCAGTCCCCGCGGTCAGGTTGGATCAACAGGCACTA-3′
Primers for RT-PCR	
aroH_F	5′-ACTGACGAACTCCGTACTGC-3′
aroH_R	5′-TCGCTTATCTTCACCATTCA-3′
aroF^{wt}_F	5′-AGACGCGCTGAATAACGTAC-3′
aroF^{wt}_R	5′-ATCGACGAGCATATTCCAGA-3′
pheA_F	5′-AAGCCCTCTACAAATTTGCC-3′
pheA_R	5′-GTTGGAGCCCTGGTCAA-3′
pheA^{fbr}_F	5′-GCGCTGGATGAAAAATTATT-3′
pheA^{fbr}_R	5′-GCTTTACCGAGCGTAATTAA-3′

RT reverse transcription

[a] Underlining indicates restriction enzyme sites; bold indicates the location of the Shine-Dalgarno sequence added artificially

with agitation (200 rpm). The overnight cell culture was diluted into Epo medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, 10 g l^{-1} NaCl, 4 g l^{-1} isonicotinic acid hydrazide, 25 g l^{-1} glycine, 0.1 % Tween 80) to an optical density at 600 nm (OD_{600}) of 0.3 and cultured at 200 rpm and 30 °C until the OD_{600} reached 0.9. The culture was then chilled on ice for 10 min, harvested by centrifugation (4,000 *g*, 10 min) and washed four times with 15 ml ice-cold 10 % (v/v) glycerol; the cells were re-suspended in 0.2 ml 10 % (v/v) glycerol, frozen and stored as aliquots at −70 °C. For electro-transformation, the aliquots of competent cells were first thawed on ice, and 1–5 μl DNA was added. The mixture was transferred to a cold electroporation cuvette (gap 0.1 cm) and electroporated at 1.8 kV using a 10-ms (approx.) pulse. Immediately after electroporation, 1 ml LBHIS media (5 g l^{-1} tryptone, 5 g l^{-1} NaCl, 2.5 g l^{-1} yeast extract, 18.5 g l^{-1} Brain Heart infusion powder, 91 g l^{-1} sorbitol) was added to the cuvette, and the contents were first mixed and then transferred to a 2-ml sterile Eppendorf tube. The mixture was then incubated at 30 °C for 2 h before being plated on LBHIS agar containing the appropriate antibiotics.

RNA extraction

Cells were harvested (2,000 *g*, 5 min) and washed twice with double distilled water and stored at −80 °C until RNA preparation. Total RNA from the untreated control and D-limonene-treated cells with and without exogenous ergosterol was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA was quantified and checked in a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) at 260 and 280 nm. The integrity of the isolated RNA was verified using an automated electrophoresis system (Bio-Rad, Hercules, CA).

Culture conditions and medium

Corynebacterium glutamicum strains were grown at 30 °C in LBG medium. Where necessary, an antibiotic, such as ampicillin (100 μg ml^{-1}), kanamycin (50 μg ml^{-1}) or chloramphenicol (34 μg ml^{-1}), was supplemented to the medium. A 10 % (v/v) inoculum of an overnight culture (18 h) was used for further studies. To induce the expression of the various genes carried by the plasmids,

isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM. Flask cultivations were carried out in 20 ml of medium in a 250-ml Erlenmeyer flask medium at 30 °C with agitation at 200 rpm.

The seed medium contained (g l^{-1}) glucose (35), corn steep liquor (35), $(NH_4)_2SO_4$ (5), urea (2), KH_2PO_4 (1), $MgSO_4{\cdot}7H_2O$ (0.5); the pH was 7.2. The shake flask fermentation medium contained (g l^{-1}) glucose (90), $(NH_4)_2SO_4$ (25), corn steep liquor (8), KH_2PO_4 (1), $MgSO_4$ (0.5), $CaCO_3$ (20); the pH was 7.2.

RT-PCR assay

cDNA was synthesized from 5 μg of total RNA using the Primescript ® RT Reagent Kit Perfect Real Time (Takara, Dalian, China), and the cDNA obtained was used as the template in the subsequent PCR assay. All primer sequences are given in Table 1. The efficiency and specificity of the primers were determined from dilution experiments and melting curves, respectively. RT-PCR experiments were performed using PrimeSTAR® HS DNA Polymerase (Takara), and the parameters for PCR cycling were: denaturation at 95 °C for 5 min, degeneration at 95 °C for 30 s, annealing at 65 °C for 30 s, extension at 72 °C for the associated time (1 kb min^{-1}), final extension at 72 °C for 10 min, and heat preservation at 12 °C.

SDS-PAGE analysis

Analysis by SDS-PAGE was performed in 10 % acrylamide gels, and the proteins were visualized by staining with Coomassie brilliant blue R-250. About 1.0 OD_{600} of cell culture was obtained after 12 h of induction. Whole cells were harvested at 4,000 *g* for 10 min and then washed third times with double distilled water. The cells were then resuspended in 40 μl lysozyme (20 mM) and lysed at 37 °C for 1 h, following which 10 μl of the 5× loading buffer was added for the SDS-PAGE analysis.

Analysis of fermentation parameters

The cell concentration was measured at OD_{600} on a spectrophotometer-722 (Third Analytical Instrument Factory, Shanghai, China) after the appropriate dilution. To determine the concentration of intracellular metabolites, 5 ml of cell culture was rapidly transferred into a centrifuge tube containing 20 ml of precooled glycerol-NaCl (glycerol and 13.5 g l^{-1} NaCl solution; volume ratio 1:1) and the mixture centrifuged (10,000 *g*, 3 min, −19 °C). The cells were then resuspended in 1 ml of precooled 50 % methanol solution in a centrifuge tube and the centrifuge tube placed in liquid nitrogen for 2–3 min and then thawed on ice; the freezing–thawing procedures were repeated three times. The supernatant obtained after centrifugation was either directly analyzed by high-performance liquid chromatography (HPLC) to determine the concentration of metabolites or preserved at −80 °C. For shikimate analysis, 5 ml of cell culture was centrifuged (7,000 *g*, 10 min), and the supernatant filtered through a filtration membrane (diameter 25 mm; pore size 0.22 μm. The filtered sample was analyzed on an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) which was equipped with a 250 × 4.6-mm ZORBAX SB-Aq column (Agilent Technologies), a standard G 1329A autosampler (Agilent Technologies) and a G13158 diode array detector (DAD) (Agilent Technologies). Na_2HPO_4 (0.138 mol l^{-1}) and acetonitrile (1 %, v/v) adjusted to pH 2.0 with phosphoric acid were used as the mobile phase at a flow rate of 1.0 ml min^{-1}. The detection wavelength was 210 nm, and the column temperature was maintained at 35 °C.

To determine the concentration of amino acids, 5 ml of cell culture or intracellular supernatant was centrifuged (10,000 *g*, 10 min) and the supernatant was diluted with trichloroacetic acid (5 g l^{-1}) and filtered through a membrane (pore size 0.22 μm). Amino acids were precolumn derivatized by *o*-phthaldialdehyde (OPA) and then analyzed on an Agilent 1100 HPLC system (Agilent Technologies) equipped with a reverse-phase column (Zorbax Eclipse-AAA) and an UV detector at 338 nm according to the procedure established by Henderson et al. [11].

Results

Single overexpression of the committed enzymes driving more flux to L-Phe

In *C. glutamicum*, the synthesis of L-Phe occurs via the shikimate pathway and the branch acid pathway. To evaluate the rate-limiting steps of the L-Phe synthesis pathways, we first examined the committed genes *aroG*, *aroF*, *pheA*, $aroF^{wt}$ and $pheA^{fbr}$ (Table 1). As shown in Table 3, under specific conditions, strains *C. glutamicum* ATCC 13032, *C. glutamicum* 19G (*aroG*), *C. glutamicum* 19F (*aroF*), *C. glutamicum* $19F^{wt}$ ($aroF^{wt}$), *C. glutamicum* 19A (*pheA*) and *C. glutamicum* $19A^{fbr}$ ($pheA^{fbr}$) accumulated L-Phe to levels of 0.34 ± 0.03, 0.43 ± 0.01, 0.56 ± 0.06, 0.88 ± 0.06, 0.27 ± 0.06 and 1.34 ± 0.02 g l^{-1}, respectively. Clearly, single overexpression of the mutated gene $aroF^{wt}$ or $pheA^{fbr}$ significantly improved L-Phe production. Compared with L-Phe, L-Tyr as the end byproduct was not increased by the upregulation of $aroF^{wt}$ or $pheA^{fbr}$; to the contrary, single overexpression of $pheA^{fbr}$ resulted in a decrease of L-Tyr accumulation (Table 3), indicating that more carbon flux was driven to L-Phe.

Table 3 Comparison of L-phenylalanine, L-tyrosine and shikimate production in different strains

Strains	L-Phe (g l^{-1})	L-Tyr (g l^{-1})	Shikimate (g l^{-1})
C. glutamicum ATCC 13032	0.34 ± 0.03	0.260 ± 0.02	0.29 ± 0.08
C. glutamicum 19G	0.43 ± 0.01	0.06 ± 0.05	0.69 ± 0.07
C. glutamicum 19F	0.56 ± 0.06	0.37 ± 0.04	1.54 ± 0.04
C. glutamicum 19F^{wt}	0.88 ± 0.06	0.27 ± 0.01	5.67 ± 0.05
C. glutamicum 19A	0.27 ± 0.06	0.32 ± 0.02	0.32 ± 0.04
C. glutamicum 19A^{fbr}	1.34 ± 0.02	0.04 ± 0.07	0.28 ± 0.05

L-PHE L-phenylalanine, L-TYR L-tyrosine

Data are presented as the mean ± standard deviation (SD) ($n = 3$)

To investigate the effect of disturbance by single overexpression of the above-mentioned genes, shikimate as the committed intermediate was also analyzed. As shown in Table 3, upregulation of the upstream genes *aroG*, *aroF* or *aroF*^{wt} could lead to an apparent accumulation of shikimate which was consistent with the accumulation of L-Phe. Specially, the recombinant *C. glutamicum* 19F^{wt} (*aroF*^{wt}) increased shikimate accumulation to 5.67 ± 0.05 g l^{-1}, suggesting that other key genes must be involved downstream of the aromatic amino acid synthesis. In addition, no L-Trp was detected throughout the cultivation process.

Introduction of the wild-type *aroH* gene for improving L-Phe production

By comparing the shikimate pathway (Fig. 1), we found that in *E. coli*, the DS enzyme is encoded by three genes (*aroG*, *aroF* and *aroH*). However, in *C. glutamicum*, it is encoded by only two genes, namely, *aroG* and *aroF*. This difference led us to hypothesize that heterogenous overexpression of the wild-type *aroH* gene in *C. glutamicum* should be active because of the natural strict regulation of the L-Trp branch pathway. As expected, introduction of the wild-type *aroH* gene increased L-Phe, L-Tyr and shikimate accumulation to 0.99 ± 0.06, 0.44 ± 0.02 and 6.80 ± 0.09 g l^{-1}, respectively (Fig. 2). Furthermore, no detectable L-Trp was accumulated during cultivation. To demonstrate the high levels of L-Phe that accumulated, we added L-Trp into the culture to study its effect on L-Phe accumulation. As shown in Fig. 2, the addition of 1 g l^{-1} L-Trp dramatically decreased L-Phe and shikimate accumulation to 0.44 ± 0.06 and 1.24 ± 0.08 g l^{-1}, respectively. At the same time, the L-Tyr level also decreased from 0.44 ± 0.02 to 0.31 ± 0.04 g l^{-1}. When the intracellular concentrations of L-Phe, L-Trp and L-Tyr were assayed, we found that L-Phe, L-Trp and L-Tyr had been maintained at milligram levels without any obvious change (data not shown), suggesting that majority of aromatic amino acids were secreted extracellular.

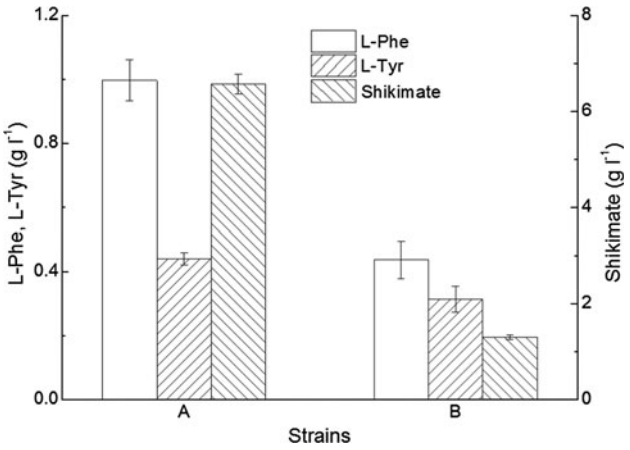

Fig. 2 Accumulation of metabolites by the recombinant *C. glutamicum* 19H (*aroH* from *E. coli*) in the absence of L-tryptophan (*l-Trp*) (**a**) and following the addition of 1.0 g l^{-1} L-Trp to the culture (**b**). Data are presented as the mean ± standard deviation (SD) ($n = 3$)

Production of L-Phe by overexpression of *aroH* and *pheA*^{fbr}

Based on these results, we constructed and comparatively investigated the recombinant *C. glutamicum* 19A^{fbr}H (*pheA*^{fbr}, *aroH*) and *C. glutamicum* 19A^{fbr}F^{wt} (*pheA*^{fbr}, *aroF*^{wt}). As shown in Fig. 3, Co-overexpression of *pheA*^{fbr} and *aroH* resulted in a substantial increase in L-Phe (4.64 ± 0.09 g l^{-1}) accumulation and a remarkable decline of shikimate (3.2 ± 0.05 g l^{-1}), which confirmed the important role of the mutated *pheA*^{fbr}. Correspondingly, *C. glutamicum* 19A^{fbr}F^{wt} accumulated L-Phe and shikimate up to levels of 4.46 ± 0.06 and 2.89 ± 0.06 g l^{-1}, respectively. By comparison, we found that co-overexpression of *pheA*^{fbr} and *aroH*, or of *pheA*^{fbr} and *aroF*^{wt} had no effect on L-Tyr accumulation, which further demonstrated that a large proportion of the carbon source was directed to L-Phe. Furthermore, L-Trp was still not detectable during the whole cultivation process.

To characterize the pathway we had engineered, we studied the expression levels of the key enzymes. At the

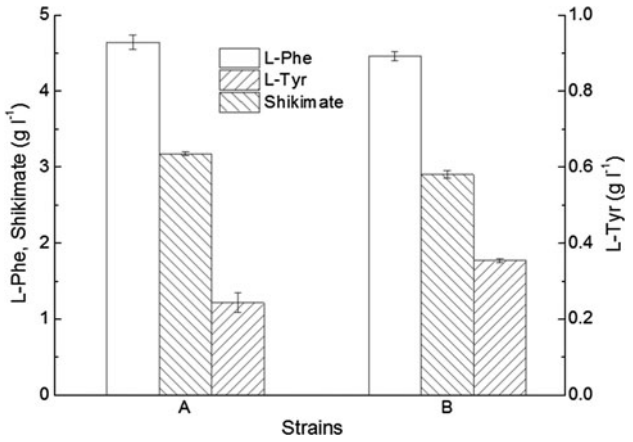

Fig. 3 Accumulation of metabolites by co-overexpression of *pheA*^{fbr} and *aroF*^{wt}, and *pheA*^{fbr} and *aroH*. **a** The recombinant *C. glutamicum* 19A^{fbr}H, **b** the recombinant *C. glutamicum* 19A^{fbr}F^{wt}. Data are presented as the mean ± SD ($n = 3$)

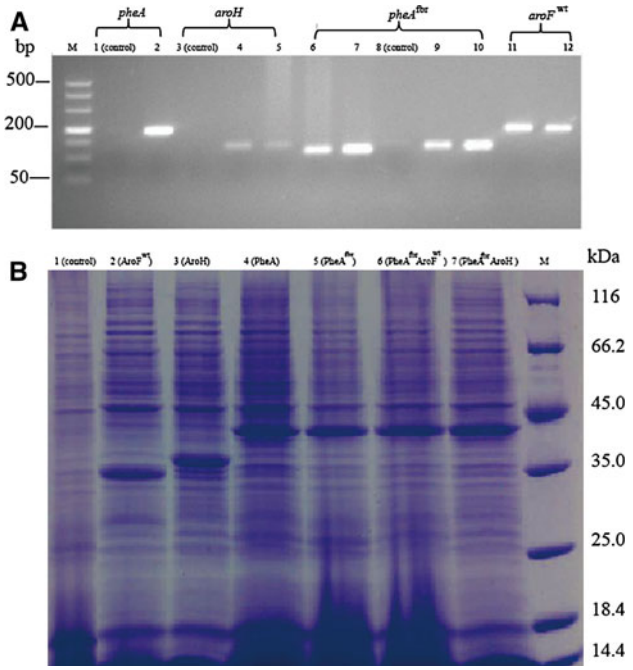

Fig. 4 Analysis of the transcript level of key enzymes in recombinant *C. glutamicum* by reverse transcription (RT)-PCR and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue staining of protein products. **a** RT-PCR. *Lanes*: *M* DL 500 DNA ladder, *1* control (*pheA*), *2 C. glutamicum* 19A (*pheA*), *3* control (*aroH*), *4 C. glutamicum* 19H (*aroH*), *5 C. glutamicum* 19A^{fbr}H (*aroH*), *6 C. glutamicum* 19A^{fbr} (*pheA*^{fbr}), *7 C. glutamicum* 19A^{fbr}H (*pheA*^{fbr}), *8* control (*pheA*^{fbr}), *9 C. glutamicum* 19A^{fbr} (*pheA*^{fbr}), *10 C. glutamicum* 19A^{fbr}F^{wt} (*pheA*^{fbr}), *11 C. glutamicum* 19F^{wt} (*aroF*^{wt}), *12 C. glutamicum* 19A^{fbr}F^{wt} (*aroF*^{wt}). **b** SDS-PAGE. *Lanes 1 C. glutamicum* ATCC 13032, *2 C. glutamicum* 19F^{wt}, *3 C. glutamicum* 19H, *4 C. glutamicum* 19A, *5 C. glutamicum* 19A^{fbr}, *6 C. glutamicum* 19A^{fbr}F^{wt}, *7 C. glutamicum* A^{fbr}H, *M* molecular weight marker

transcriptional level, all of the committed genes *pheA*, *pheA*^{fbr}, *aroH*, *aroF* and *aroF*^{wt} investigated were successfully transcribed (Fig. 4a); this result is consistent with our cultivation results. However, an unbalanced expression level was observed by SDS-PAGE analysis. When the *aroF*^{wt}, *aroH*, *pheA* and *pheA*^{fbr} genes were individually overexpressed, a distinct band of each enzyme was detected (Fig. 4b). However, when *pheA*^{fbr} and *aroH* or *pheA*^{fbr} and *aroF*^{wt} were co-overexpressed within one operon, no distinct bands of AroH and AroF were detected (Fig. 4b).

Discussion

In nature, the biosynthetic pathway of amino acids is strictly regulated (generally by feedback inhibition) in *C. glutamicum*. As a result, modification of the committed enzymes to relieve or eliminate the feedback inhibition by the end-products is crucial to increasing the target compound [29, 32]. In this study, the wild-type *aroH* gene from *E. coli* was cloned into *C. glutamicum* to optimize the shikimate pathway for producing L-Phe.

Many studies have been carried out in *E. coli*, resulting in a substantial improvement in the production of L-Phe [1, 8, 13, 33]. When we first investigated the committed genes involved in L-Phe production, we found that single overexpression of the native wild-type *aroG*, *aroF* and *pheA* genes had no obvious effect on the yield of L-Phe (Table 3), further demonstrating that the synthesis of L-Phe in *C. glutamicum* is extremely tightly regulated. To the contrary, when either the mutated *aroF*^{wt} (*E. coli*) or *pheA*^{fbr} (*E. coli*) gene was introduced into *C. glutamicum*, the titer of L-Phe was dramatically increased (Table 3), indicating that feedback inhibition to DS (encoded by *aroF*^{wt}) or CM-PDT [12] (encoded by *pheA*^{fbr}) had occurred. Subsequent studies on the overexpression of the mutated *aroF*^{wt} from *E. coli* in *C. glutamicum* revealed that shikimate was increased up to a high level (Table 3), demonstrating that other key enzymes are present in downstream of the pathway.

In *E. coli*, the DS enzyme encoded by *aroH* is inhibited by feedback from L-Trp, contributing 1 % to DS enzyme activity [13, 27]. In contrast, no *aroH* gene is naturally present in *C. glutamicum*, and thus the introduction and overexpression of the wild-type *aroH* gene from *E. coli* may be beneficial to the production of L-Phe by *C. glutamicum*. As expected, introduction of *aroH* achieved a substantial increase in L-Phe production without L-Trp accumulation, thereby demonstrating the complete inhibition of the native branch pathway for L-Trp synthesis (Fig. 1). The capacity of the *aroH* gene and the strategy of co-overexpression of the committed enzymes have been studied [19, 29, 32, 33]. As predicted, we found that co-

overexpression of the rate-limiting enzymes DS and CM-PDT yielded a high titer of L-Phe (Fig. 3). Moreover, compared with *C. glutamicum* $19A^{fbr}F^{wt}$, *C. glutamicum* $19A^{fbr}H$ showed the better property, which revealed that although *aroF* was mutated, the release of feedback inhibition may be incomplete in vivo. To modulate and balance the pathway flux, we also investigated the expression levels of the key genes at the transcriptional and translational levels, respectively. At the transcriptional level, all of the genes studied were successfully transcribed (Fig. 4a). However, an unbalanced expression level was observed in the SDS-PAGE analysis. When two genes were co-expressed with one operon, the expression level of the gene further away from the promoter declined sharply and no obvious band was detected (Fig. 4b). Similar results have been reported in *E. coli* [19]. Consequently, to further increase the titer of the targeted product, subtle regulation of the committed genes might be crucial [5, 28].

In conclusion, for a comparative analysis of the committed enzymes involved in the aromatic amino acid synthesis pathway in *C. glutamicum* and *E. coli*, we used the wild-type gene *aroH* from *E. coli* for improving L-Phe production. As predicted, the introduction and overexpression of this gene in *C. glutamicum* remarkably increased L-Phe production by *C. glutamicum*. Compared with $aroF^{wt}$, the *aroH* gene from *E. coli* was shown to be more effective for L-Phe production. Moreover, the observed unbalanced expression levels of the genes within one operon suggested that fine control of the committed enzymes will be very important for improving L-Phe production. The knowledge gained through this study will provide further insights into novel pathway engineering of *C. glutamicum* for L-Phe production.

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