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• BRIEF REPORTS •

Integration of *E. coli aro*G-*phe*A tandem genes into *Corynebacterium glutamicum tyr*A locus and its effect on L-phenylalanine biosynthesis

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Abstract

AIM: To study the effect of integration of tandem *aro*G-*phe*A genes into the *tyr*A locus of *Corynebacterium glutamicum* (*C. glutamicum*) on the production of L-phenylalanine.

METHODS: By nitrosoguanidine mutagenesis, five *p*-fluorophenylalanine (FP)-resistant mutants of *C.glutamicum* FP were selected. The *tyr*A gene encoding prephenate dehydrogenase (PDH) of *C.glutamicum* was amplified by polymerase chain reaction (PCR) and cloned on the plasmid pPR. Kanamycin resistance gene (Km) and the P_{BF}-*aro*G-*phe*A-T (GA) fragment of pGA were inserted into *tyr*A gene to form targeting vectors pTK and pTGAK, respectively. Then, they were transformed into *C.glutamicum* FP respectively by electroporation. Cultures were screened by a medium containing kanamycin and detected by PCR and phenotype analysis. The transformed strains were used for L-phenylalanine fermentation and enzyme assays.

RESULTS: Engineering strains of *C.glutamicum* (Tyr$^-$) were obtained. Compared with the original strain, the transformed strain *C. glutamicum* GAK was observed to have the highest elevation of L-phenylalanine production by a 1.71-fold, and 2.9-, 3.36-, and 3.0-fold in enzyme activities of chorismate mutase, prephenate dehydratase and 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase, respectively.

CONCLUSION: Integration of tandem *aro*G-*phe*A genes into *tyr*A locus of *C. glutamicum* chromosome can disrupt *tyr*A gene and increase the yield of L-phenylalanine production.

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INTRODUCTION

L-phenylalanine is one of the essential amino acids for humans. Its applications range from feed to food,and pharmaceutical products. such as *p*-fluorophenylalanine are used as anti-tumour drugs. At present, L-phenylalanine biosynthesis genes have been well characterized and the enzymology of L-phenylalanine biosynthesis has been extensively investigated[1-10]. In bacteria, the biosynthesis of aromatic amino acids starts from condensation reaction of central carbon intermediates such as phosphoenol pyruvate (PEP) and erythrose-4-phosphate (E4P) to form 3-deoxy -D-arabino -heptulosonate-7-phosphate (DAHP), which is catalyzed by DAHP synthase (DS). DAHP is then converted to chorismate, the branch point of aromatic amino acid biosynthesis. L-phenylalanine is synthesized from chorismate by three continuous steps catalyzed by chorismate mutase (CM), prephenate dehydratase (PD) and aromatic-amino-acid transaminase (AT). *aro*G[1-5] and *phe*A[6-10] are two key genes in L-phenylalanine biosynthesis. In *E. coli*, they encode the 3-deoxy-D-arabinoheptulosonate -7-phosphate synthase (DS) and chorismate mutase/prephenate dehydratase (CM/PD), respectively. However, in *Corynebacterium glutamicum* (*C. glutamicum*), CM and PD are encoded by two different genes *phe*B and *phe*A[10]. The over-expression of *aro*G and *phe*A can increase the yield of L-phenylalanine biosynthesis in *E. coli* or in *Corynebacteria* by shuttle vectors[11-17]. *tyr*A encoding prephenate dehydrogenase(PDH) is a key gene in L-tyrosine biosynthesis branch pathway[18]. DS and CM of *C. glutamicum* can be synergistically inhibited by L-tyrosine and L-phenylalanine. Every step from DAHP to chorismate is repressed weakly by L-tyrosine. In addition, PD is strongly inhibited by L-phenylalanine and L-tryptophan, L-tyrosine stimulates PD activity and restores the enzyme activity inhibition by L-phenylalanine and L-tryptophan[19]. These regulations seem to result in balanced synthesis of L-tyrosine and L-phenylalanine (Figure 1). Therefore, elimination of feedback inhibition of L-phenylalanine on the key enzymes and deletion of *tyr*A gene can improve the yield of L-phenylalanine biosynthesis. There are successful examples by using homologous recombination technology to introduce genes into chromosome[20-23].

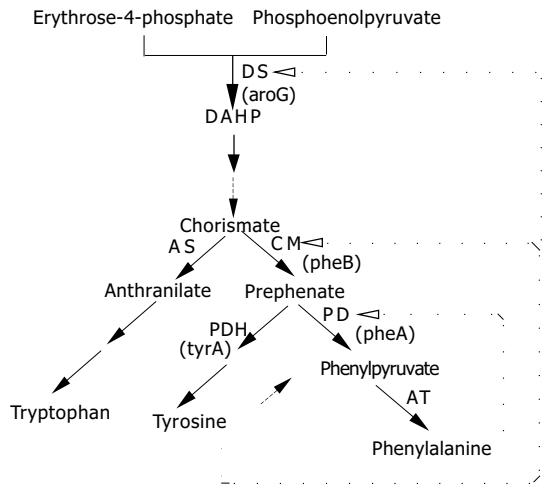

Figure 1 Pathway and primary regulations of phenylalanine biosynthesis in *C. glutamicum* ⊲---- indicates feedback inhibition, - - - ▶ indicates activation.

In this study, we used nitrosoguanidine(NTG) to treat *C. glutamicum*. Mutants resistant to FP were isolated in a

minimal medium plate with FP and named *C.glutamicum* FP. The tandem *aro*G-*phe*A[15] genes obtained from *E. coli* resistant to FP were inserted into the chromosome of *C.glutamicum* FP by a double-exchange homologous recombination between the fragments of *tyr*A gene on targeting vectors and the *tyr*A gene on the chromosome of *C.glutamicum* FP. Enzyme activities and L-phenylalanine biosynthesis in the engineered strains of *C.glutamicum* KM and *C.glutamicum* GAK were analyzed.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. Minimal medium[15] (MM) and NB (LB added with 10 g/L beef extract) were used for culture *C. glutamicum*. The medium for *C. glutamicum* fermentation was reported previously[16]. LB was used for growth of *E. coli*. When required, appropriate antibiotics were added to the suitable concentrations. Solid media were made by the addition of agar at the concentration of 15 g/L.

Chemicals

T_4 DNA ligase, restriction enzymes, gel extract kit and DNA blunting kit were obtained from Takara Co. NTG and the reagents used in the enzyme assays were purchased from Sigma Co. They were used according to the instructions of their manufacturers.

Isolation of L-phenylalanine analogue resistant mutants

C. glutamicum cells were grown in NB supplemented with 20 mg/L nalidixic acid until log phase, and collected by centrifugation, followed by two washes with phosphate buffered saline (pH6.0).The cells were then resuspended in 500 μL of 9 g/L NaCl containing 5 mg of NTG and kept on shaking at 30 ℃ for another 30 min. The cells were collected by centrifugation followed by two washes before suspension in 9 g/L NaCl. Appropriate aliquots of the suspension were spread on the minimal agar plate containing different concentrations of *p*-fluorophenylalanine (*p*-FP) for mutant screening.

Preparation and manipulation of DNA

Chromosomal DNA of *C. glutamicum* was extracted as described preciously[10]. Manipulation of plasmid DNA was performed as described by Sambrook *et al*.[24].

Construction of recombinant plasmids

Primers of *tyr*A and Km were designed according to the sequences in GenBank (NC_003450 and AF012346). Primer P1: 5'-G GCTGCAGCAGGCCATCCTCTTCAGTGTTCA-3' and primer P2: 5'-CCCA AGCTTCGAGGGTTTCAGCGTGGATG-3' were designed for amplifying *tyr*A gene carrying *Pst*I and *Hin*dIII restriction enzyme sites, respectively. Primer P3: 5'-GCGGAAT TCACCGGAATTGCCAGCTG-3' and primer P4: 5'-GCGGAAT TCTGCAGTTATCAGAAGAACTCGTCAA G-3' were designed for amplifying Km gene carrying *Eco*RI and *Pst*I sites, respectively. The primer P5 for PCR detection was 5'-CCCA AGCTTAGG AATCGCCTGAGAATCATCA-3'. Construction of recombinant plasmids was performed as shown in Figure 2.

Electroporation of linear DNA into C. glutamicum

Targeting vectors were digested by *Hin*d III and the linear DNA was obtained by gel extraction. Then, they were denatured by 1 mol/L NaOH at 37 ℃ for 10 min, quickly placed on ice before the same volume of 1 mol/L HCl was added to neutralize the alkali. The method of Molenaar[25] was used to introduce denatured targeting vectors into *C.glutamicum* FP.

Selection and identification of the recombinant strain

After electroporation, *C.glutamicum* was incubated in 1mL NB medium containing 0.5 mol/L sucrose at 30 ℃ by shaking (100 r/min) for 1 h, then the culture was plated on selective NB medium containing 0.5 mol/L sucrose, 10 μg/mL nalidixic acid and 15 μg/mL kanamycin for 96 h. The strains on resistant plates were singled out and inoculated on NB with 100 μg/mL ampicillin, 15 μg/mL kanamycin and NB with 15 μg/mL kanamycin, respectively; Clones ($Ap^s Km^r$) were detected by PCR using primers pair P3 and P5. PCR products were sequenced.

Detection of Tyr phenotype

The strain cultures were washed three times with sterile physiological saline and plated on MM plates containing 2 g/L glucose and 1 mol/L L-tyrosine or other amino acids to detect the Tyr^- auxotroph.

Enzymatic activities assay

Crude cell lysates used for enzymatic activity assays were prepared as described previously[15]. Total protein level was determined according to the method of Bradford[26]. DS activity was assayed as described previously[15]. CM activity was determined as described by Xia *et al*.[27]. PD activity was assayed as preciously described[28].

Fermentation and analysis of phenylalanine

Transformants of *C. glutamicum* were obtained by PCR and phenotype analysis. L-phenylalanine fermentation of them was carried out in the shaking flask and the yields of L-phenylalanine biosynthesis were determined as described by Zeng *et al*.[15].

Table 1 Strains and plasmids

Strains or plasmids	Relevant characteristics	Sources or references
E.coli JM110	Strain for vectors construction	Our laboratory
C.glutamicum	Nx^r, original strainmutagenesis	ATCC
C.glutamicum FP	FP^r, from *C.glutamicum* ATCC13032	This work
C.glutamicum KM	*C. glutamicum* FP inserted with Km gene	This work
C.glutamicum GAK	*C. glutamicum* FP inserted with GAK	This work
pBluscript SK (pSK)	Ap^r, vector for gene cloning	Our laboratory
pPR	Ap^r, pBR322 derivative, suicidal vector	Our laboratory
pGA	Km^r, carrying P_{BF}-*aro*G-*phe*A-T fragment	Ref.15
pT	pPR carrying *tyr*A gene	This work
pTK	pT carrying Km gene(tyrA::Km)	This work
pSKGAK	pSK carrying GAK fragment	This work
pTGAK	pT carrying GAK fragment (tyrA::GAK)	This work

FP^r: resistance to *p*-fluorophenylalanine; Nx^r: resistance to nalidixic acid; Ap^r: resistance to ampicillin; Km^r: resistance to kanamycin; ATCC: American Type Culture Collection. $P_{BF.}$ promoter from *C.glutamicum*; T: terminator of gene32 in T_4 phage. GAK: P_{BF}-*aro*G-*phe*A-T-Km fragment.

RESULTS

Isolation of L-phenylalanine analogue resistant mutants

Five mutants resistant to *p*-FP up to 3 mg/mL were picked out on MM plates after NTG mutagenesis. In those mutants, the feedback inhibition on the key enzymes in biosynthesis pathways was released from phenylalanine. The mutant with the highest yield of phenylalanine was named *C.glutamicum* FP and used as the recipient strain for electroporation (unpublished data). *C.glutamicu m* FP was detected by PCR with primers P1 and P2. The sequence of PCR products was consistent with *tyr*A of *C.glutamicum* ATCC 13032.

Construction of recombinant plasmids

Km (950 bp) and *tyr*A (1067 bp) genes were amplified by PCR and they were verified by sequencing. pPR vector and *tyr*A gene were digested with *Pst*I and *Hin*d III and ligated to form pT. pT was digested with *Bst*EII, and then blunted. Km gene and P_{BF}-*aro*G-*phe*A-T fragment from pGA plasmids were subcloned into pSK vector, then digested with *Pst* I and *Hin*d III to get GAK fragment (P_{BF}-*aro*G-*phe*A-T-Km). This resulting fragment and the Km gene were inserted into the pT to form targeting vectors pTGAK and pTK, respectively. The detailed process refers to Figure 2.

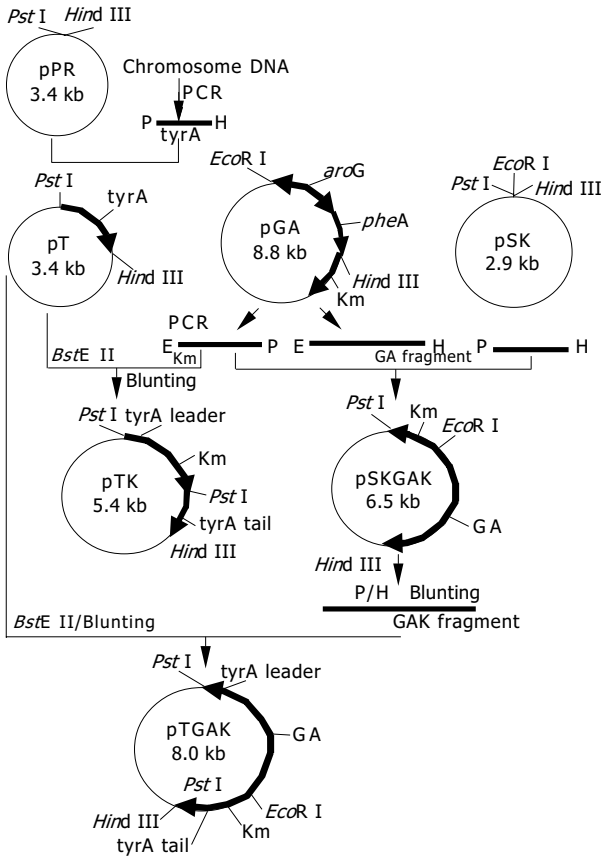

Figure 2 Construction of recombinant plasmids. E: *Eco*RI; P: *Pst*I; H: *Hin*d III; GA: P_{BF}-*aro*G-*phe*A-T fragment; GAK: P_{BF}-*aro*G-*phe*A-T-Km fragment.

Identification of transformants

Because primer P5 was located at the end of *tyr*A gene, it only existed on the chromosome of *C.glutamicum*, but not on the targeting vector. Only when the Km gene was inserted into the chromosome of *C.glutamicum*, could the product be obtained by PCR using the primer pair P3 and P5 (Figure 3). The expected PCR products of 1570 bp are shown in Lane 3 and Lane 5 of Figure 4. Since a *Pst*I restriction site was introduced into Km gene in foreside (Figure 2), the obtained PCR products could be digested by *Pst*I to produce two fragments whose sizes (950 bp and 620 bp) accorded to the sizes of Km gene and an expanded *tyr*A tail as shown in Lane 2 and Lane 4 of Figure 4. Sequencing of the 1 570 bp fragment demonstrated it consisted of Km gene and a 620 bp fragment including *tyr*A tail (unpublished data). The strains obtained by PCR detection were named *C. glutamicum* KM (inserted with Km) and *C. glutamicum* GAK (inserted with GAK).

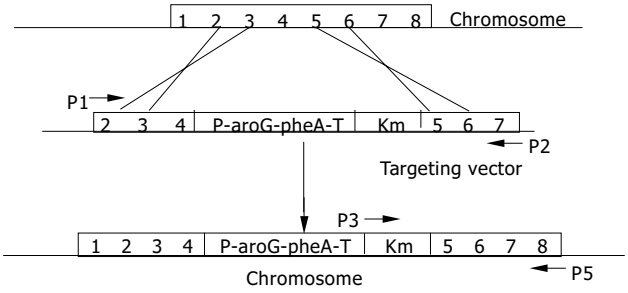

Figure 3 Insertion of P_{BF}-*aro*G-*phe*A-T-Km fragment of pTGAK into chromosome *tyr*A locus of *C. glutamicum* by double-exchange homologous recombination and detection of ligated DNA fragment Km-*tyr*A tail on chromosome by PCR amplification with P3 and P5.

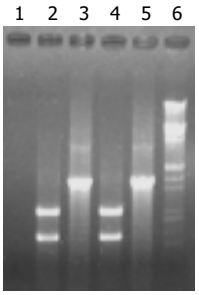

Figure 4 DNA detection of transformants by PCR amplification and *Pst*I digested PCR products. Lane 1: control for PCR; lane 2: *Pst*I digesting product of lane 3; lane 3: PCR detection of *C. glutamicum* KM; lane 4, *Pst*I digesting product of lane 5; lane 5: PCR detection of *C. glutamicum* GAK; lane 6: Markers, *Eco*RI / *Hin*d III digesting λ phage DNA.

Detection of Tyr auxotroph

As shown in Figure 5, *C. glutamicum* GAK, in which *tyr*A was disrupted by inserting a GAK fragement, only grew on the MM with L-tyrosine, but not on MM and MM with L-tyrptophan or L-phenylalanine plates. This result indicated that *C. glutamicum* GAK was an auxotroph requiring L-tyrosine for growth. Here, we demonstrated that the pathway of tyrosine biosynthesis at steps from prephenate to 4-hydroxyphenlpyruvate was disrupted, and the function of prephenate dehydrogenase was inactivated successfully.

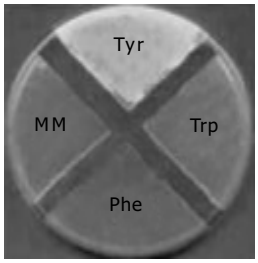

Figure 5 Auxanogram detection of *C. glutamicum* (Tyr$^-$). MM: *C. glutamicum* GAK on MM; Tyr: *C.glutamicum* GAK on MM+Tyr; Phe: *C.glutamicum* GAK on MM+Phe; Trp: *C.glutamicum* GAK on MM+Trp.

Enzyme assay

As shown in Figure 1, in the pathway of L-phenylalanine biosynthesis, there were three key enzymes, PD, CM and DS, encoded by *phe*A, *phe*B and *aro*G, respectively in *C.glutamicum.* The results of enzyme activity in *C.glutamicum* are listed in Table 2. The activity of the original strain was used as the control. Compared to the original strain, the relative activities of three enzymes (CM, PD and DS) in *C.glutamicum* FP were 1.73-, 2.37- and 1.93-fold higher and in *C.glutamicum* GAK were 2.9-, 3.36- and 3.0-fold higher respectively. The relative activities of these enzymes in *C.glutamicum* KM (Km^r FP^r) were similar to those in *C.glutamicum* FP (FP^r) respectively. The results indicated that the enzyme activities were increased differently by inhibiting the release of L-phenylalanine feedback using FP^r selection and the integration of tandem genes *aro*G-*phe*A into chromosome of *C.glutamicum.* At the same time, it also proved that the homologous recombination was successful.

Table 2 Relative activities of CM, PD and DS compared with the original strains

Strains	Enzymes		
	CM	PD	DS
C. glutamicum	1.0	1.0	1.0
C. glutamicum FP	1.73	2.37	1.93
C. glutamicum KM	1.74	2.30	1.95
C. glutamicum GAK	2.90	3.36	3.00

The enzyme activity of *C.glutamicum* was used as the standard, *i.e.* the relative activity of 1. CM: chorismate mutase; PD: prephenate dehydratase; DS: 3-deoxy-D-arabinoheptulosonate-7-phosphate synthase.

Measurement of phenylalanine yields

Recombinant strains were shaked in 25 mL of fermentation medium with 0.5 mol/L L-tyrosine in 250 mL flask at 30 ℃ for 72 h. The average yield of three measurements is shown in Table 3. From these data, we could find inhibition of the release of feedback contributed to increase the yield of L-phenylalanine obviously, the yield was increased 42%. On the other hand, interruption of the L-tyrosine branch pathway also increased 9% of the yield. Integration of the tandem genes *aro*G-*phe*A made 20% improvement. The total yield was increased 71%. The highest yield was added up to 3.97 g/L.

Table 3 Yields of L-phenylalanine biosynthesis in flask fermentation (mean±SD)

Strains	*C.glutamicum*	*C.glutamicum* FP	*C.glutamicum* KM	*C.glutamicum* GAK
Yields (g/L)	2.20±0.15	3.12±0.17	3.32±0.12	3.76±0.21
Relative yields	1	1.42	1.51	1.71

The phenylalanine yield of the original stain was used as the standard, *i.e.* the relative yield of 1.

DISCUSSION

The results of this study showed that interruption of the branch pathway of L-tyrosine biosynthesis could accumulate intermediates for L-phenylalanine synthesis and integration of the tandem *aro*G-*phe*A encoding DS, CM and PD from *E. coli* into the *C.glutamicum* chromosome was also favorable to L-phenylalanine synthesis due to the improvement of key enzyme activities in biosynthesis pathways. These two factors contributed to the increase of 30% yield in L-phenylalanine biosynthesis. By increasing the precursors for phenylalanine synthesis, interruption of the tyrosine branch pathway could decrease L-tyrosine synthesis, and inhibit L-phenylalanine synthesis because L-tyrosine could stimulate prephenate dehydratase activity and restore the enzyme activity inhibited by L-phenylalanine and L-tryptophan. Ours results also indicated that mutagenesis by NTG was still an effective method for screening phenylalanine-produing strains. This method could effectively to improve the amino acid production in this study. The strains integrated with Km gene kept their resistance to kanamycin after cultured in medium without antibiotics for 96 h (unpublished data). Compared to the strains carrying plasmids[15-17], strains integrated with heterologous genes had lower yields of L-phenylalanine but a higher stability. Further mutagenesis and application of strong promoters may be useful to increase the yield of L-phenylalanine of engineering strains. Further investigations are necessary to understand the mechanism of aromatic amino acid biosynthesis.

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