**Integration of *E. coli* aroG-pheA tandem genes into *Corynebacterium glutamicum* tyrA locus and its effect on L-phenylalanine biosynthesis**

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**Abstract**

AIM: To study the effect of integration of tandem aroG-pheA genes into the tyrA 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 tyrA gene encoding prephenate dehydrogenase (PDH) of *C. glutamicum* was amplified by polymerase chain reaction (PCR) and cloned on the plasmid pP1. Kanamycin resistance gene (Km) and the polymerase chain reaction (PCR) and cloned on the plasmid dehydrogenase (PDH) of *C. glutamicum* FP-resistant mutants of *C. glutamicum* were obtained. Then, a gene encoding prephenate dehydrogenase (PDH) was inserted into tyrA 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-arabinohexulosonate-7-phosphate synthase, respectively.

CONCLUSION: Integration of tandem aroG-pheA genes into tyrA locus of *C. glutamicum* chromosome can disrupt tyrA 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-aminoo-acid transaminase (AT). aroG[11-15] and pheA[16-20] are two key genes in L-phenylalanine biosynthesis. In *E. coli*, they encode the 3-deoxy-D-arabinohexulosonate-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 pheB and pheA[21]. The over-expression of aroG and pheA can increase the yield of L-phenylalanine biosynthesis in *E. coli* or in *Corynebacterium* by shuttle vectors[19,22]. tyrA encoding prephenate dehydrogenase (PDH) is a key gene in L-tyrosine biosynthesis branch pathway[23]. 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[24]. 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 tyrA gene can improve the yield of L-phenylalanine biosynthesis. There are successful examples by using homologous recombination technology to introduce genes into chromosome[25-28].

Figure 1 Pathway and primary regulations of phenylalanine biosynthesis in *C. glutamicum*

In this study, we used nitrosoguanidine(NTG) to treat *C. glutamicum*. Mutants resistant to FP were isolated in a
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 [13] (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 [14]. 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

T4 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 °C 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 previously [15]. Manipulation of plasmid DNA was performed as described by Sambrook et al. [16].

Construction of recombinant plasmids

Primers of tyrA and Km were designed according to the sequences in GenBank (NC_003450 and AP012346). Primer P1: 5'-GCTGCAAGGCGGCACTTTCAGGTTCATA-3' and primer P2: 5'-CCCAGGCTTGGGTTTTCAACGCTGGATG-3' were designed for amplifying tyrA gene carrying PstI and HindIII restriction enzyme sites, respectively. Primer P3: 5'-GGCGGAATTCACCGGAATTGCGACGTCGTAAG-3' and primer P4: 5'-GGCGGAATTCGGACGCTTGTGATTGACGTCAGAAAG-3' were designed for amplifying Km gene carrying EcoRI and PstI sites, respectively. The primer P5 for PCR detection was 5'-CCAGTTAGGAAATGCCTGAAGATCTCATA-3'. Construction of recombinant plasmids was performed as shown in Figure 2.

Electroproportion of linear DNA into C. glutamicum

Targeting vectors were digested by HindIII and the linear DNA was obtained by gel extraction. Then, they were denatured by 1 mol/L NaOH at 37 °C 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 [17] was used to introduce denatured targeting vectors into C. glutamicum FP.

Selection and identification of the recombinant strain

After electroproportion, C. glutamicum was incubated in 1 mL NB medium containing 0.5 mol/L sucrose at 30 °C 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, Km) 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 [18]. Total protein level was determined according to the method of Bradford [19]. DS activity was assayed as described previously [18]. CM activity was determined as described by Xia et al. [20]. PD activity was assayed as previously described [21].

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. [22].

Table 1 Strains and plasmids

| Strains or plasmids | Relevant characteristics | Sources or references |
|---------------------|--------------------------|----------------------|
| E.coli JM110        | Strain for vectors construction | Our laboratory |
| C.glutamicum        | Nxr, original strainmutagenesis | ATCC |
| C.glutamicum FP     | FP, 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 |
| pBluescript SK (pSK)| Ap⁺, vector for gene cloning | Our laboratory |
| pPR                 | Ap⁺, pBR322 derivative, suicidal vector | Our laboratory |
| pGA                 | Km⁺, carrying Pty-aroG-phenA-T fragment | Ref.15 |
| pT                  | pFR carrying tyrA 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⁺: resistance to p-fluorophenylalanine; Nxr⁺: resistance to nalidixic acid; Ap⁺: resistance to ampicillin; Km⁺: resistance to kanamycin; ATCC: American Type Culture Collection. Pty⁺ promoter from C.glutamicum; T: terminator of gene32 in T4 phage. GAK: Pty-aroG-phenA-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 phe (unpublished data). The strains obtained by PCR detection were named C. glutamicum KM (inserted with Km) and C. glutamicum GAK (inserted with GAK).

Construction of recombinant plasmids

Km (950 bp) and tyrA (1067 bp) genes were amplified by PCR and were verified by sequencing. pPR vector and tyrA gene were digested with Pst I and Hind III and ligated to form pT. pT was digested with BstE II, and then blunted. Km gene was digested with Pst I and Hind III, and they were verified by sequencing. pPR vector and targeting vector. Only when the Km gene was inserted into the chromosome of C. glutamicum was named C. glutamicum ATCC 13032.

Identification of transformants

Because primer P5 was located at the end of tyrA 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 PstI restriction site was introduced into Km gene in foreside (Figure 2), the obtained PCR products could be digested by PstI to produce two fragments whose sizes (950 bp and 620 bp) accorded to the sizes of Km gene and an expanded tyrA tail as shown in Lane 2 and Lane 4 of Figure 4. Sequencing of the 1570 bp fragment demonstrated it consisted of Km gene and a 620 bp fragment including tyrA 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 2 Construction of recombinant plasmids. E: EcoRI; P: PstI; H: Hind III; GA: PαG-aroG-pheA-T fragment; GAK: PαG-aroG-pheA-T-Km fragment.

Detection of Tyr auxotroph

As shown in Figure 5, C. glutamicum GAK, in which tyrA gene was inactivated successfully, only grew on the MM with L-tyrosine, but not on MM and MM with L-tryptophan 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-hydroxyphenylpyruvate was disrupted, and the function of prephenate dehydrogenase was inactivated successfully.

Figure 3 Insertion of PαG-aroG-pheA-T-Km fragment of pTGAK into chromosome tyrA locus of C. glutamicum by double-exchange homologous recombination and detection of ligated DNA fragment Km-tyrA tail on chromosome by PCR amplification with P3 and P5.

Figure 4 DNA detection of transformants by PCR amplification and PstI digested PCR products. Lane 1: control for PCR; lane 2: PstI digesting product of lane 3; lane 3: PCR detection of C. glutamicum KM; lane 4, PstI digesting product of lane 5; lane 5: PCR detection of C. glutamicum GAK; lane 6: Markers, EcoRI/Hind III digesting λ phage DNA.

Identification of transformants

Because primer P5 was located at the end of tyrA 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 PstI restriction site was introduced into Km gene in foreside (Figure 2), the obtained PCR products could be digested by PstI to produce two fragments whose sizes (950 bp and 620 bp) accorded to the sizes of Km gene and an expanded tyrA tail as shown in Lane 2 and Lane 4 of Figure 4. Sequencing of the 1570 bp fragment demonstrated it consisted of Km gene and a 620 bp fragment including tyrA tail (unpublished data). The strains obtained by PCR detection were named C. glutamicum KM (inserted with Km) and C. glutamicum GAK (inserted with GAK).
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 pheA, pheB and aroG, 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 (KmFP) were similar to those in C. glutamicum FP (FP) respectively. The results indicated that the enzyme activities were increased differently by inhibiting the release of L-phenylalanine feedback using FP selection and the integration of tandem genes aroG-pheA 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 | Activities (fold) |
|--------------------|---------|-------------------|
| C. glutamicum      | CM      | 1.0               |
|                    | PD      | 1.0               |
|                    | DS      | 1.0               |
| C. glutamicum FP   | CM      | 1.73              |
|                    | PD      | 2.37              |
|                    | DS      | 1.93              |
| C. glutamicum KM   | CM      | 1.74              |
|                    | PD      | 2.30              |
|                    | DS      | 1.95              |
| C. glutamicum GAK  | CM      | 2.90              |
|                    | PD      | 3.36              |
|                    | DS      | 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 °C 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 aroG-pheA 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 | C. glutamicum | C. glutamicum |
|--------------------|---------------|---------------|---------------|---------------|
|                    | FP            | KM            | 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 strain 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 aroG-pheA 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-producing 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 plasmid[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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