RESEARCH NOTES

Phenylalanine biosynthesis in *Brevibacterium lactofermentum* using *Escherichia coli* genes *pheA*, *aroG* and *tyrB* *

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Abstract Genetic engineering technology to increase the production of L-phenylalanine was used in the study. Three genes encoding the key enzymes involved in the biosynthesis of L-phenylalanine were utilized, in which the gene *aroG* encodes 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase (DS); the gene *pheA* encodes bifunctional enzyme of chorisate mutase (CM) and prephenate dehydratase (PD); and the gene *tyrb* encodes aminotransferase (AT). The three genes were amplified by polymerase chain reaction (PCR) from the genome of the *E. coli* mutant strains resistant to fluro-DL-phenylalanine and inserted into the cloning vectors. Then, they were expressed in *E. coli* and *Brevibacterium lactofermentum* in a tandem arrangement. The expressed enzymes had high activities in the host cells.

Keywords: ***aroG*, *pheA and tyrB* genes, co-expression, L-phenylalanine biosynthesis.**

L-phenylalanine is one of the essential amino acids for human nutrition, and is used as a component of amino acid infusions for medical purposes. For the past 15 years, the biosynthesis of L-phenylalanine has attracted more and more attentions, due to the increasing demands of the dipeptide sweetener Aspartame.

As an aromatic amino acid, L-phenylalanine can be biosynthesized by microorganisms and plants, but not by humans and animals. From glucose, by Embden Meyerhof pathway (EMP) and hexose monophosphate pathway (HMP), microorganisms take in phosphoenolpyruvate and erythrose-4-phosphate, and then with seven enzyme-catalyzed steps chorismate is synthesized from phosphoenolpyruvate and erythrose-4-phosphate. From there, three separate branches diverge toward their final products, one of which is L-phenylalanine. The regulatory patterns about this complex biosynthesis pathway have been reported[1]. The 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase (DS), chorismate mutase (CM) and prephenate dehydratase (PD) are the three key enzymes feedback-inhibited by L-phenylalanine. The aminotransferase (AT) is an important enzyme which catalyzes phenylpyruvate into phenylalaine at the last step.

With the genes for amino acid biosynthesis well-characterized, it is possible to use the well-developed recombinant DNA technology to produce phenylalanine in bacteria. Besides the classical genetic approaches, the development of a host-vector system for modifying the phenylalanine-producing

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bacteria is of importance. It has been reported that large amounts of phenylalanine production can be achieved by amplifying the rate-limiting enzymes in the pheylalanine-producing strain[2]. The metabolic conversion from tryptophan to phenylalanine or tyrosine by amplifying enzymes of CM and PD in *Corynebacterium glutamicum* KY10865 was also reported[3]. Introduction of the genes encoding DS and tryptophan-biosynthetic enzymes into *C. glutamicum* KY10894 resulted in a marked increase (54%) in yield of tryptophan[4]. Employing resting cells of TA-overproducing *E. coli* K-12 strain for phenylalanine production resulted in molar conversion yield of 55%[5]. We have previously reported the phenylalanine production by using tyrosine auxotroph and desensitized mutant resistant to fluorophenylalanine of *Brevibacterium flavum*[6], and developed a shuttle vector in both *E. coli* and *B. flavum* as the expressing system[7]. The engineering pathway of aromatic compound-producing strain via eliminating feedback inhibition, deleting branched pathway and by over-expressing desired genes was also reported recently[8~11].

In this study, we used native genes of *E. coli* as potentially favorable genetic material for improving a phenylalanine-producing mutant of *Brevibacterium*. The three genes of *aroG*, *pheA* and *tyrB* encoding DS, CM, PD and TA respectively were cloned from the *E coli* mutant strains resistant to fluorophenylalanine and expressed in phenylalanine producer of *B. lactofermentum*. Activities of the related enzymes and their effect on the phenylalanine biosynthesis were analyzed, and the genetic engineering strains were obtained.

1 Materials and methods

1.1 Strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Table 1 Bacterial strains and plasmids

Strains or plasmids	Characteristics	Source or references
E. cloi XL-1-blule-G	$TA^{r\,a)}$ FP^{r} (donor of *aro*G)	mutated from XL-1-blule
E. coli MV1184-A	TA^{r} $FP^{r\,b)}$ (donor of *phe*A)	mutated from MV1184
E. coli MV1184-B	$TA^{r}FP^{r}$ (donor of *tyrB*)	mutated from MV1184
E. coli P2392	Recipient strain for clonin and expression	Ref. [10]
Brevibacterium flavum F75	Nutrient-deficiency strain (*phe*A arxotroph)	Ref. [7]
Brevibacterium lactofermentum 2731	$Nx^{r\,c)}$ FP^{r} recipient strain (phe producer)	mutated from ATCC13869
Plasmid pUC118	$Ap^{r\,d)}$ (gene cloning vector)	stock in our laboratory
Plasmid $p\lambda P_R$	Ap^{r} (gene expressing vector)	Ref. [8]
Plasmid pCZ	$Km^{r\,e)}$ (gene expressing vector)	Ref. [1]

a) TA^{r}, resistance to thienylalanine; b) FP^{r}, resistance to fluorophenylalanine; c) Nx^{r}, resistance to nalidixic acid; d) Ap^{r}, resistance to ampicillin; e) Km^{r}, resistance to kanamycin.

1.2 Culture of bacteria

Strains of *E. coli* were grown in Luria broth (LB)[8]. Strains of *Brevibacterium* were grown in a complete medium according to Ref.[6] and genetic engineering strains were grown in a production medium (PM)[6]. When required, antibiotics were added to the media at the final concentrations of 1 mg/mL for TA; 1 mg/mL for FP; 20 mg/mL for Nx; 100 μg/mL for ampicillin; 25 μg/mL for

kanamycin.

1.3 Preparation and manipulation of DNA

Chromosomal DNA and plasmid DNA in *E. coli* were extracted as described by Sambrook et al[12]. Chromosomal DNA and plasmid DNA in *B. lactofermentum* were isolated as described by Lei et al[7].

1.4 Construction of recombinant plasmids and engineering strains

According to the DNA sequences of *aroG*, *pheA*, and *tyrB* described in Ref. [13 ~ 15], the primers were designed and synthesized using chemical methods. The genes were amplified by PCR as described in Ref. [1] from the *E. coli* strains in Table 1, and cloned into pUC118, then transferred into pλ PR and pCZ, respectively, in different tandem arrangements. Construction of recombinant plasmids and engineering strains was performed (Fig. 1). Recombinant plasmids were transformed into *E. coli* or *Brevibacterium* for expression as described by Jiang et al.[8] and Lei et al.[7], respectively.

1.5 Enzyme assays

The cells containing cloned genes were harvested by centrifugation at 6000 r/min for 10 min at 4℃, and were washed and suspended in 50 mmol/L potassium phosphate buffer (pH 7.0). Crude cell extracts were prepared by sonic disruption of washed cells. Cellular debris were removed by centrifugation at 8000 r/min for 25 min at 4℃ to obtain the supernatants. The supernatants were fractionated with ammonium sulfate, and the crude extracts were dialyzed against the above potassium phosphate buffer for 12 h, and then used for the enzyme assays. DS activity in crude cell extracts was measured by the method of Jiang et al.[8]. CM and PD activities were measured by the method of Fan et al.[9]. AT activity was measured by the method of Peng et al.[10].

1.6 Fermentation and analysis of phenylalanine

Fermentation was carried out according to the method of Fan et al.[6]. Analysis of fermentation products was carried out using the method of Lei et al.[11].

2 Results and discussion

2.1 Gene cloning and construction of recombinant DNA

The construction of engineered strains harboring three genes is shown in Fig 1. The obtained recombinant plasmids and strains are listed in Table 2.

2.2 Enzyme activities

Table 2 shows the enzyme activities in the cells of *E. coli* and *B. lactofermentum* harboring different constructs. Three genes of *aroG*, *pheA and tyrB* could be expressed in both types of the host cells as we expected. Activities of the expressed enzymes were increased significantly. For example, the increased activities of DS, CM, PD and AT in *E. coli* P2392/pλPR-GAB were 7.5-, 11.9-, 6.

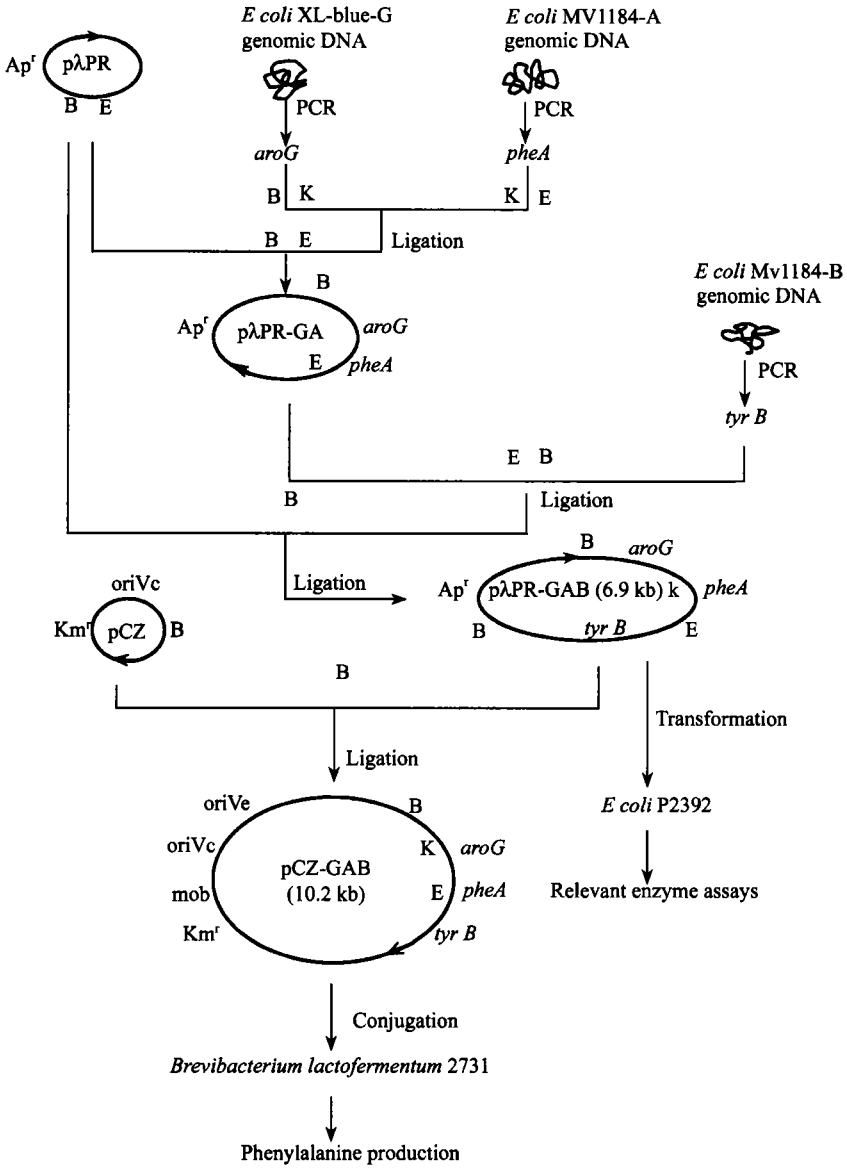

Fig. 1 Construction of plasmids and strains harboring genes of *aroG*, *pheA* and *tyrB*. B, *Bam*HI; E, *Eco*RI; K, *Kpn* I.

8- and 2.3-fold, and in *B. lactofermentum* 2731/pCZ-GAB were 4.4-, 4.5-, 2.8- and 3.2-fold those of the host cells without recombinant genes.

2.3 Analysis of phenylalanine production in the engineered strains

Table 3 shows a comparison of phenylalanine production of the engineered strains with that of the host strain. All 40 engineered strains had increased phenylalanine production, 1.85-fold higher on average, among which the yields of six strains (8, 12, 13, 19,38 and 40 in Table 3) were over 2 fold that of the host strain.

Table 2 Specific activities of enzymes in the engineered strains as compared with those of host strains

Strains/plasmids	Coloned genes and linked orders	Relative activities of enzymes[a)]			
		DS	CM	PD	AT
E coli P2392/pλPR	control	1.0	1.0	1.0	1.0
E coli P2392/pλPR-AB	*pheAtyrB*	–	13.1	6.7	3.1
E coli P2392/pλPR-GA	*aroGpheA*	4.5	4.4	2.3	–
E coli P2392/pλPR-GAB	*aroGpheAtyrB*	7.5	11.9	6.8	2.3
B lactofermentum 2731/pCZ	control	1.0	1.0	1.0	1.0
B lactofermentum 2731/pCZ-AB	*pheAtyrB*	–	7.9	4.1	4.6
B lactofermentum 2731/pCZ-GA	*aroGpheA*	6.8	6.7	3.3	–
*B lactofermentum*2731/pCZ-GAB	*aroGpheAtyrB*	4.4	4.5	2.8	3.2

a) Activity of enzyme in the host strain is as a standard, i.e. relative activity is 1.0. —, not tested.

Table 3 Phenylalanine production of engineered strain *B. lactofermentum*/pCZ-GAB (%)

Engineered strain No.	Yields	Engineered strain No.	Yields	Engineered strain No.	Yields	Engineered strain No.	Yields
1	197	11	185	21	188	31	168
2	179	12	204	22	192	32	165
3	180	13	212	23	170	33	168
4	196	14	199	24	181	34	157
5	170	15	197	25	152	35	181
6	173	16	184	26	171	36	188
7	171	17	192	27	196	37	179
8	212	18	191	28	191	38	200
9	197	19	212	29	121	39	196
10	196	20	199	30	179	40	212

The yield of host strain *B. Lactofermentum* 2731 was taken as a standard (i.e.100%).

3 Discussion

We have reported that the genes of *aroG*, *pheA* and *tyrB* from *E. coli*, encoding four key enzymes involved in the biosynthesis pathway of phenylalanine, could be co-expressed in both *B. flavum* 1311[1] and *E. coli* P2392[8]. In this work, the three genes with different tandem arrangements in the shuttle plasmid pCZ could be also co-expressed in *B. lactofermentum* 2731 which was driven from *Brevibacterium lactofermentum* ATCC13869. Activities of the relevant enzymes were increased greatly; thus production of phenylalanine in engineered strain was increased 2-fold as high as that of the host strain. These results demonstrated that the engineering pathway based on the metabolic mechanism of phenylalanine is useful for increasing the end product in the biosynthesis pathway. However, it is essential to adopt a selective pressure with antibiotics in case the cells lose the recombinant plasmid, and a procedure to ensure plasmid stability should be taken. With more research, the metabolic pathway engineering strain will be applicable to the production of phenylalanine on a large scale.

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