Co-expression of five genes in E coli for L-phenylalanine in Brevibacterium flavum

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AIM: To study the effect of co-expression of ppsA, pckA, aroG, pheA and tyrB genes on the production of L-phenylalanine, and to construct a genetic engineering strain for L-phenylalanine.

METHODS: ppsA and pckA genes were amplified from genomic DNA of E. coli by polymerase chain reaction, and then introduced into shuttle vectors between E. coli and Brevibacterium flavum to generate constructs pJ N2 and pJ N5. pJ N2 was generated by inserting ppsA and pckA genes into vector pCZ; whereas pJ N5 was obtained by introducing ppsA and pckA genes into pCZ-GAB, which was originally constructed for co-expression of aroG, pheA and tyrB genes. The recombinant plasmids were then introduced into B. flavum by electroporation and the transformants were used for L-phenylalanine fermentation.

RESULTS: Compared with the original B. flavum cells, all the transformants were showed to have increased five enzyme activities specifically, and have enhanced L-phenylalanine biosynthesis ability variably. pJ N5 transformant was observed to have the highest elevation of L-phenylalanine production by a 3.4-fold. Co-expression of ppsA and pckA increased activity of DAHP synthetase significantly.

CONCLUSION: Co-expression of ppsA and pckA genes in B. flavum could remarkably increase the expression of DAHP synthetase; Co-expression of ppsA, pckA, aroG, pheA and tyrB of E. coli in B. flavum was a feasible approach to construct a strain for phenylalanine production.

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INTRODUCTION

L-phenylalanine, one of the essential amino acids in human, is used as a major component of amino acid in infusion clinically. For the past two decades, biosynthesis of L-phenylalanine has attracted more and more attentions due to the increasing demand of Aspartame, a dipeptide sweetener containing L-phenylalanine.

Production of L-phenylalanine by microbes has clear advantages over chemical synthesis, e.g., the biological processes are more environmentally sound and utilize renewable resources. In bacteria, the biosynthesis of aromatic amino acids starts from condensation reaction of central carbon intermediates phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) to form 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP), which is catalyzed by DAHP synthetase (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). In E. coli, aroG and tyrB genes encode DS and AT, respectively, whereas CM and PD are encoded by a single gene pheA.

Since the genes coding for amino acid biosynthesis are well characterized in E. coli and other microbes, it is possible to make metabolic pathway engineering via recombinant DNA approach to increase the productivity of phenylalanine in bacteria. As reported previously, introduction of a single gene pheA into Corynebacterium glutamicum resulted in a 35 % increase of L-phenylalanine production. In our previous work, co-expression of aroG, pheA and tyrB in Brevibacterium lactofermentum with pCZ-GAB gave a 2-fold increase in L-phenylalanine yield. On the other hand, elevation of intracellular levels of the precursor PEP is considered to be essential to channel more carbon flux into aromatic flux in order to get higher yield of L-phenylalanine. In E. coli, two enzymes are involved in the formation of PEP. PEP synthetase (PpsA) catalyzes the synthesis of PEP from pyruvate by transphosphorylation reaction, whereas PEP carboxykinase (PckA) catalyzes the synthesis of PEP from oxaloacetate by decarboxylation reaction. PpsA and PckA are encoded by ppsA gene and pckA gene, respectively. Overexpression of ppsA gene in E coli has been shown to elevate DAHP level by a 1.9-fold. PckA over-expression in E coli cells showed a 20 % increase in molar conversion yields for L-phenylalanine production.

In this study, the ppsA and pckA genes in E coli were amplified from genomic DNA by polymerase chain reaction (PCR), and then introduced into a B. flavum-E. coli shuttle vector with arroG, both pheA and tyrB genes were used as operons. The constructs were transformed into B. flavum for L-phenylalanine fermentation, and the specific activities of each enzyme as well as the L-phenylalanine yield were measured.

MATERIALS AND METHODS

Bacterial strains and plasmids

All the strains and plasmids used in this study are listed in Table 1. E coli XL1-Blue-G and B. flavum 311 are mutants resistant to phenylalanine analogue fluorophenylalanine. XL1-Blue-G was used as donor of ppsA and pckA.
Table 1 Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics | Source or reference |
|-------------------|--------------------------|---------------------|
| E. coli XL1-BlueG | Fp' (donor of ppsA, pckA) | Ref. 28             |
| E. coli P2392     | (strain for expression)   | Stored by our lab   |
| B. flavum 311    | Nx, Fp (strain for expression and fermentation) | Stored by our lab |
| pSK-P            | Ap (E. coli expressing vector) | This study |
| pJN2             | pcZ inserted with ppsA, pckA and pckA tandemly | This study |
| pJN5             | pcZ-GAB inserted with tandem ppsA, pckA | This study |
| pSK-Par          | pBluescript SK- inserted with promoter Pbf | Structured by our lab, unpublished |
| pcZ              | Km (E. Flav -E. coli shuttle vector) | Stored by our lab |
| pcZ-GAB          | pcZ inserted with tandem arC, pheA, tyrB | Ref. 1 |
| pJN2             | pcZ inserted with tandem ppsA, pckA | This study |
| pJN5             | pcZ-GAB inserted with tandem ppsA, pckA | This study |

Fp', resistance to fluorophenylalanine; Nx, resistance to nalidixic acid; Ap', resistance to ampicillin; Km, resistance to kanamycin.

Media and growth conditions

E. coli and transformants containing plasmid were grown at 37 °C in Luria-Bertani medium. B. flavum and plasmid-containing transformants were grown in complete medium at 31 °C for DNA manipulation and expression, and were grown in production medium for fermentation as described previously[11]. Media were supplemented with the following antibiotics as required: fluorophenylalanine (1 mg/mL), nalidixic acid (10 µg/mL), ampicillin (100 µg/mL), kanamycin (20 µg/mL).

Construction of recombinant plasmids

Primers for amplification of ppsA gene were synthesized according to Ref. 29 with addition of restriction enzyme sites of EcoRI for forward primer (5' - GCATGAATTCGATGTC AACAATGGCTCGT-3') and KpnI for reverse primer (5' - GCATGTACCCGATTCGATGCAGGT-3'). Primers for amplification of pckA gene were designed according to Ref. 30 with addition of restriction enzyme sites of KpnI for forward primer (5' - GCATGTTACCATATTGCGTAAGGAGCAGTG-3') and HindIII for reverse primer (5' - TACGGAAGCTTATCCACGAAACCAGTG-3'). The genes were amplified by PCR and cloned on pBlueScript II SK+ and transferred on to expression vector pλPbf in a tandem arrangement as showed in Figure 1. The fragment containing tandem ppsA and pckA was then inserted into shuttle vector pcZ and pCZ-GAB to obtain pJN2 and pJN5.

Enzymatic activity assay

Crude lysates used for enzymatic activity assays were prepared as described previously[1]. The total protein level was determined by the method of Bradford[12]. PpsA activity was determined by method[13] with modification. In brief, each PpsA assay mixture contained 1.5 µmol/L pyruvate, 10 µmol/L ATP, 10 µmol/L MgCl₂, 100 µmol/L Tris-HCl (pH 8.0), and 200 µL crude lysates. The reaction was terminated by adding 0.3 mL 100 g/L TCA and 0.1 mL 1 g/L 2,4-dinitrophenylhydrazine, and was monitored by measuring the consumption of pyruvate at 520 nm. PckA activity was determined as publication[31] with modification. In brief, each PckA assay mixture contained 10 µmol/L PEP, 50 µmol/L NaHCO₃, 4 µmol/L ADP, 80 µmol/L MgCl₂, 100 µmol/L Tris-HCl (pH 7.5), and 100 µL crude lysates. The reaction was terminated by adding 0.75 mL ethanol and 20 µL 20 g/L Fast
Violet B Salt, and was monitored at 520 nm. DS and AT activities were assayed as described previously\cite{28}. CM activity was determined as the method of Xia\cite{35}. PD activity was assayed as the method of Ref. 35.

**Fermentation and analysis of phenylalanine**

Fermentation of *B. flavum* 311 was carried out and the fermentation yields of L-phenylalanine were determined by the method of Ref. 1.

**RESULTS**

**Expression of ppsA and pckA genes in transformed E. coli cells**

The *ppsA* and *pckA* genes were amplified from *E. coli* genomic DNA by PCR and were then subsequently cloned onto pBluescript II SK(+) plasmid at corresponding restriction sites. Minor point mutations were detected on the amino acid sequences of *PpsA* and *PckA* protein as determined by DNA sequencing (data not shown). These two genes were expressed in *E. coli* to confirm its bioactivities.

The expression vectors were constructed based on vector pλP2 to either express a single gene or co-express the two genes as an operon. The constructs were transformed into in *E. coli* P2392 cells and the protein profiles of transformants were analyzed by SDS-PAGE (Figure 2). Distinct protein bands corresponding to the molecular weights of *PpsA* and *PckA* were detected on SDS-PAGE as shown in Figure 2. The relative specific activities of the transformants were also determined (Table 2). Independent expression of *ppsA* and *pckA* genes resulted in increase in specific enzymatic activities of the corresponding enzymes by 4.2- and 1.5-fold, respectively. Whereas in co-expression of *ppsA* and *pckA* genes, the increases in specific enzymatic activities were 2.1-fold and 1.3-fold, a slightly lower than that of independent expression. The results suggested that the two genes amplified by PCR had the normal enzymatic activities.

![Figure 2 SDS-PAGE analysis of total proteins of *E. coli* P2392 cells harboring different recombinant plasmids. Lane 1, total protein of *E. coli* P2392 cells; lane 2, harboring pλP2; lane 3, harboring pλP2-pps; lane 4, protein markers; lane 5, harboring pλP2-pck; lane 6, harboring pλP2-pck. Arrows indicate molecular weight of the protein markers and the positions of *PpsA* and *PckA*.

**Table 2** The relative specific enzymatic activities in *E. coli* P2392 harboring different constructs

| Strain/ plasmid | Relative enzymatic activities | PpsA | PckA | DS |
|-----------------|-----------------------------|------|------|----|
| E. coli P2392/ pλP2 | 1                          | 1    | 1    | 1  |
| E. coli P2392/ pλP2-pps | 5.2                        | 1.0  | 1.8  |    |
| E. coli P2392/ pλP2-pck | 1.0                        | 2.5  | 1.5  |    |
| E. coli P2392/ pλP2-2p | 3.1                        | 2.3  | 2.1  |    |

When we looked at the enzymatic activities of DS in different transformants, we found that either single-gene expression or co-expression of *ppsA* and *pckA* genes could induce elevated expression of DS by 0.5 to 1.1-fold.

**Enzymatic activities in transformed *B. flavum* 311 cells**

To attempt metabolite pathway engineering in *B. flavum*, a host strain for L-phenylalanine production, shuttle vectors pJN2 and pJN5 were constructed and introduced into *B. flavum* 311 cells by electroporation. The specific enzymatic activities were measured for each transformants as summarized in Table 3. For the pJN5-harboring transformant, all of the six specific enzymatic activities had increased. The pJN2-harboring transformant showed higher specific activities for *PpsA* and *PckA* than pJN5-harboring transformant as expected. Again, a significant increase in DS activity was observed, though *aroG* gene was not expressed by pJN2, which was very similar to the results of pλP2-2p in *E. coli* P2392. Unexpectedly, all of the DS, AT and CM/PD enzymatic activities of pJN5-harboring transformant were higher than that of pCZ-GAB-harboring transformant, since the copy number of pJN5 in *B. flavum* was lower than that of pCZ-GAB (data not shown).

![Table 3 Relative specific enzymatic activities in *B. flavum* 311 harboring different constructs](image)

**Phenylalanine production in transformed *B. flavum* 311 cells**

To investigate the effect of enhanced enzymatic activities on L-phenylalanine production, the *B. flavum* 311 transformants harboring different constructs were subject to fermentation under conditions described in Materials and Methods, and the L-phenylalanine yield was determined. As shown in Table 4, pJN5-harboring transformant had a 2.4-fold increase in phenylalanine yield compared with the original *B. flavum* 311 cells, which had the highest phenylalanine yield among all the transformants. On the other hand, the effect of *ppsA* and *pckA* genes on L-phenylalanine yield was strictly limited with only a 0.3-fold increase. Phenylalanine yield of the pCZ-GAB-harboring transformant was almost equal to that of pJN5, implying that biosynthesis of L-phenylalanine was mainly determined by *aroG*, *tyrB* and *pheA* genes.

![Table 4 Phenylalanine production of *B. flavum* harboring different constructs](image)

**DISCUSSION**

The metabolic pathway engineering of microorganisms has been considered as the most promising approach to achieve high yield of fermentation products. Over-expressing of genes...
playing important roles in biosynthesis pathway, and introducing of special genes isolated from other organisms by genetic manipulation, are major approaches for metabolic pathway engineering. Elevation of PpaA and PckA levels in bacterial cells usually leads to the accumulation of PEP, a limiting precursor of biosynthesis of L-phenylalanine. Therefore, over-expression of ppaA and pckA genes is expected to channel more carbon flux into aromatic flux. In this study, we amplified ppaA and pckA genes from E. coli genomic DNA, and successfully expressed the two genes together with other three genes in B. flavum to investigate the effect of over-expression of these genes on biosynthesis of L-phenylalanine. Our studies revealed that expression of ppaA/pckA genes both in E. coli and in B. flavum could not only significantly elevate the enzymatic activities of PpaA/PckA, but also remarkably increase the expression of DS, which plays a central role downstream PEP in the pathway of phenylalanine biosynthesis.

As shown in Table 4, introduction of pJN2, pCZ-GAB and pJN5 into B. flavum could increase the phenylalanine yield by 0.3-, 2.0- and 2.4-fold, respectively. The differences between pJN2 and the two others are significant. A reasonable conclusion from this result is that although ppaA and pckA genes are important for accumulating PEP, they are not crucial for phenylalanine yield. The net increase in phenylalanine yield by ppaA and pckA genes when other three genes (aroG, pheA and tyrB) are over-expressed, is 13 %. Though 13 % is not a big increase, but this makes a sense for industrial scale production of L-phenylalanine. These results demonstrated feasibility to increase the phenylalanine yield by over-expressing ppaA and pckA genes in microorganisms.

Recently it was reported that the disruption of csrA gene could increase gluconeogenesis and decrease glycolysis, and thus could in turn accumulate PEP.[16-41] A strain in which the aromatic (shikimate) pathway had been optimized produced twofold more phenylalanine when csrA was disrupted. We have cloned this gene in this study and are trying to construct expression plasmid carrying csrA gene as well as other genes investigated. With further effort, metabolic pathway engineering will be finally applicable to the production of phenylalanine on a large scale.

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