P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2003;9(2):342-346
World Journal of Gastroenterology
 ISSN 1007-9327

• BASIC RESEARCH •

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

Yong-Qing Wu, Pei-Hong Jiang, Chang-Sheng Fan, Jian-Gang Wang, Liang Shang, Wei-Da Huang

Yong-Qing Wu, Chang-Sheng Fan, Jian-Gang Wang, Liang Shang, Department of Microbiology, School of Life Science, Fudan University, Shanghai 200433, China
Pei-Hong Jiang, Wei-Da Huang, Department of Biochemistry, School of Life Science, Fudan University, Shanghai 200433, China
Pei-Hong Jiang, equal contribution as the first author
Supported by the National Natural Science Foundation of China, No. 30070020
Correspondence to: Chang-Sheng Fan, Department of Microbiology, Fudan University, 220 Han Dan Road, Shanghai 200433, China. csfan@fudan.edu.cn
Telephone: +86-21-65642808 **Fax:** +86-21-55522773
Received: 2002-07-01 **Accepted:** 2002-07-26

Abstract

AIM: To study the effect of co-expression of *pps*A, *pck*A, *aro*G, *phe*A and *tyr*B genes on the production of L-phenylalanine, and to construct a genetic engineering strain for L-phenylalanine.

METHODS: *pps*A and *pck*A 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 pJN2 and pJN5. pJN2 was generated by inserting *pps*A and *pck*A genes into vector pCZ; whereas pJN5 was obtained by introducing *pps*A and *pck*A genes into pCZ-GAB, which was originally constructed for co-expression of *aro*G, *phe*A and *tyr*B 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. pJN5 transformant was observed to have the highest elevation of L-phenylalanine production by a 3.4-fold. Co-expression of *pps*A and *pck*A increased activity of DAHP synthetase significantly.

CONCLUSION: Co-expression of *pps*A and *pck*A genes in *B. flavum* could remarkably increase the expression of DAHP synthetase; Co-expression of *pps*A, *pck*A, *aro*G, *phe*A and *tyr*B of *E. coli* in *B. flavum* was a feasible approach to construct a strain for phenylalanine production.

Wu YQ, Jiang PH, Fan CS, Wang JG, Shang L, Huang WD. Co-expression of five genes in *E coli* for L-phenylalanine in *Brevibacterium flavum*. *World J Gastroenterol* 2003; 9(2): 342-346
http://www.wjgnet.com/1007-9327/9/342.htm

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[2,3].

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[4-8]. 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)[9,10]. 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*, *aro*G and *tyr*B genes encode DS and AT, respectively[11-17], whereas CM and PD are encoded by a single gene *phe*A[18-21].

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 *phe*A into *Corynebacterium glutamicum* resulted in a 35 % increase of L-phenylalanine production[22]. In our previous work, co-expression of *aro*G, *phe*A and *tyr*B in *Brevibacterium lactofermentum* with pCZ-GAB gave a 2-fold increase in L-phenylalanine yield[1]. 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[23], whereas PEP carboxykinase (PckA) catalyzes the synthesis of PEP from oxaloacetate by decarboxylation reaction[24-26]. PpsA and PckA are encoded by *pps*A gene and *pck*A gene, respectively. Overexpression of *pps*A gene in *E coli* has been shown to elevate DAHP level by a 1.9-fold[27]. PckA over-expression in *E coli* cells showed a 20 % increase in molar conversion yields for L-phenylalanine production[15].

In this study, the *pps*A and *pck*A 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 *aro*G, both *phe*A and *tyr*B 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 fluoropheylalanine. XL1-Blue-G was used as donor of *pps*A and *pck*A.

Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E.coli XL1-Blue-G	Fp^r (donor of *pps*A, *pck*A)	Ref. 28
E.coli P2392	(strain for expression)	Stored by our lab
B. flavum 311	Nx^r, Fp^r (strain for expression and fermentation)	Stored by our lab
$p\lambda P_R$	Ap^r (*E. coli* expressing vector)	Ref. 28
$p\lambda P_R$-pps	$p\lambda P_R$ inserted with *pps*A	This study
$p\lambda P_R$-pck	$p\lambda P_R$ inserted with *pck*A	This study
$p\lambda P_R$-2p	$p\lambda P_R$ inserted with *pps*A and *pck*A tandemly	This study
pSK-P_{BF}	pBluscript SK- inserted with promoter P_{BF}	Structured by our lab, unpublished
pCZ	Km^r (*B. Flavum-E.coli* shuttle vector)	Stored by our lab
pCZ-GAB	pCZ inserted with tandem *aro*G *phe*A *tyr*B	Ref. 1
pJN2	pCZ inserted with tandem *pps*A *pck*A	This study
pJN5	pCZ-GAB inserted with tandem *pps*A *pck*A	This study

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

Media and growth conditions

E. coli and transformants containing plasmid were grown at 37 ℃ in Luria-Bertani medium. *B. flavum* and plasmid-containing transformants were grown in complete medium at 31 ℃ for DNA manipulation and expression, and were grown in production medium for fermentation as described previously[1]. 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 *pps*A gene were synthesized according to *Ref.* 29 with addition of restriction enzyme sites of *EcoR*I for forward primer (5' -GCATGAATTCGATGTCC AACAATGGCTCGTC-3') and *Kpn*I for reverse primer (5' -GCATGGTACCGATTCGATTGCGATGCAGGT-3'). Primers for amplification of *pck*A gene were designed according to *Ref.* 30 with addition of restriction enzyme sites of *Kpn*I for forward primer (5' -GCATGGTACCATATTGG CTAAGGAGCAGTG-3') and *Hind*III for reverse primer (5' -TACGAAGCTTATCCAGCGAACCGTG-3'). The genes were amplified by PCR and cloned on pBlueScript II SK(+) and transferred on to expression vector $p\lambda P_R$ in a tandem arrangement as showed in Figure 1. The fragment containing tandem *pps*A and *pck*A 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[31]. PpsA activity was determined by method[32] described with modification. In brief, each PpsA assay mixture contained 1.5 μmol/L pyruvate, 10 μmol/L ATP, 10 μmol/L $MgCl_2$, 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-dinitro phenylhydrazine, and was monitored by measuring the consumption of pyruvate at 520 nm. PckA activity was determined as publication[33] with modification. In brief, each PckA assay mixture contained 10 μmol/L PEP, 50 μmol/L $NaHCO_3$, 4 μmol/L ADP, 80 μmol/L $MgCl_2$, 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

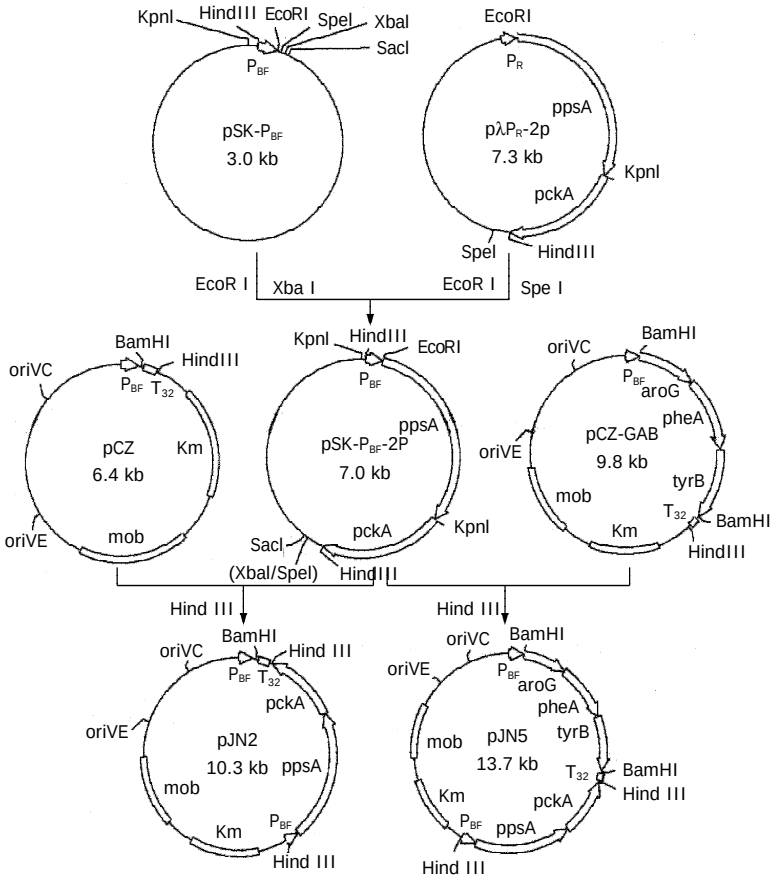

Figure 1 Construction of recombinant plasmids

Violet B Salt, and was monitored at 520 nm. DS and AT activities were assayed as described previously[28]. CM activity was determined as the method of Xia[34]. 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 $p\lambda P_R$ 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 *pps*A and *pck*A 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 *pps*A and *pck*A 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.

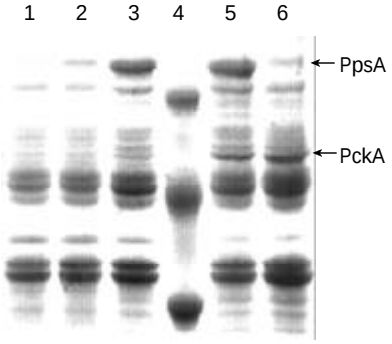

Figure 2 SDS-PAGE analysis of total proteins of *E. coli* P2392 cells harboring different recombinant plasmids. Lane 1, total protein of *E. coli* P2392 cells; lane 2, harboring $p\lambda P_R$; lane 3, harboring $p\lambda P_R$-pps; lane 4, protein markers, lane 5, harboring $p\lambda P_R$-2p; lane 6, harboring $p\lambda P_R$-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 / $p\lambda P_R$	1	1	1
E coli P2392 / $p\lambda P_R$ -pps	5.2	1.0	1.8
E coli P2392 / $p\lambda P_R$ -pck	1.0	2.5	1.5
E coli P2392 / $p\lambda P_R$ –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 *pps*A and *pck*A 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 *aro*G gene was not expressed by pJN2, which was very similar to the results of $p\lambda P_R$-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 *G.flavum* transformant was lower than that of pCZ-GAB (data not shown).

Table 3 Relative specific enzymatic activities in *B. flavum* 311 harboring different constructs

Constructs	Relative specific enzymatic activities				
	PpsA	PckA	DS	CM / PD	AT
pCZ	1	1	1	1 / 1	1
pJN2	4.2	4.5	1.9	1.0 / 1.2	1.2
pCZ-GAB	0.9	1.1	4.2	4.1 / 2.2	4.7
pJN5	2.8	3.3	6.3	4.9 / 2.5	5.5

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 *pps*A and *pck*A 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 *aro*G, *tyr*B and *phe*A genes.

Table 4 Phenylalanine production of *B. flavum* harboring different constructs

Constructs	Yield (g / L)	Relative yield
None	1.64±0.27	1
pCZ	1.59±0.29	1
pJN2	2.04±0.25	1.3
pCZ-GAB	4.83±0.18	3.0
pJN5	5.39±0.32	3.4

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 PpsA 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 *pps*A and *pckA* genes is expected to channel more carbon flux into aromatic flux. In this study, we amplified *ppsA* 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 *pps*A/*pck*A genes both in *E. coli* and in *B. flavum* could not only significantly elevate the enzymatic activities of PpsA/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 *pps*A and *pck*A genes are important for accumulating PEP, they are not crucial for phenylalanine yield. The net increase in phenylalanine yield by *pps*A and *pck*A genes when other three genes (*aro*G, *phe*A and *tyr*B) 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 *pps*A and *pck*A genes in microorganisms.

Recently it was reported that the disruption of *csr*A gene could increase gluconeogenesis and decrease glycolysis, and thus could in turn accumulate PEP[36-41]. A strain in which the aromatic (shikimate) pathway had been optimized produced twofold more phenylalanine when *csr*A was disrupted. We have cloned this gene in this study and are trying to construct expression plasmid carrying *csr*A 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.

REFERENCES

1 **Fan CS**, Jiang PH, Zeng XB, Wu YQ, Chen YQ, Huang WD. Phenylalanine biosynthesis in *Brevibacterium lactofermentum* using *Escherichia coli* genes *phe*A, *aro*G and *tyr*B. *Progress in Natural Science* 2001; **11**: 786-791

2 **Murata T**, Horinouchi S, Beppu T. Production of poly (L-aspartyl-L-phenylalanine) in *Escherichia coli*. *J Biotechnol* 1993; **28**: 301-312

3 **Tollefson L**, Barnard RJ. An analysis of FDA passive surveillance reports of seizures associated with consumption of aspartame. *J Am Diet Assoc* 1992; **92**: 598-601

4 **Petersen S**, Mack C, de Graaf AA, Riedel C, Eikmanns BJ, Sahm H. Metabolic consequences of altered phosphoenolpyruvate carboxykinase activity in *Corynebacterium glutamicum* reveal anaplerotic regulation mechanisms *in vivo*. *Metab Eng* 2001; **3**: 344-361

5 **Bongaerts J**, Kramer M, Muller U, Raeven L, Wubbolts M. Metabolic engineering for microbial production of aromatic amino acids and derived compounds. *Metab Eng* 2001; **3**: 289-300

6 **Yang YT**, Bennett GN, San KY. The effects of feed and intracellular pyruvate levels on the redistribution of metabolic fluxes in *Escherichia coli*. *Metab Eng* 2001; **3**: 115-123

7 **Delaunay S**, Uy D, Baucher MF, Engasser JM, Guyonvarch A, Goergen JL. Importance of phosphoenolpyruvate carboxylase of *Corynebacterium glutamicum* during the temperature triggered glutamic acid fermentation. *Metab Eng* 1999; **1**: 334-343

8 **Berry A**. Improving production of aromatic compounds in *Escherichia coli* by metabolic engineering. *Trends Biotechmol* 1996; **14**: 250-256

9 **Tatarko M**, Romeo T. Disruption of a global regulatory gene to enhance central carbon flux into phenylalanine biosynthesis in *Escherichia coli*. *Curr Microbiol* 2001; **43**: 26-32

10 **Gosset G**, Yong-Xiao J, Berry A. A direct comparison of approaches for increasing carbon flow to aromatic biosynthesis in *Escherichia coli*. *J Ind Microbiol* 1996; **17**: 47-52

11 **Kikuchi Y**, Tsujimoto K, Kurahashi O. Mutational analysis of the feedback sites of phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase of *Escherichia coli*. *Appl Environ Microbiol* 1997; **63**: 761-772

12 **Ger YM**, Chen SL, Chiang HJ, Shiuan D. A single Ser-180 mutation desensitizes feedback inhibition of the phenylalanine-sensitive 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase in *Escherichia coli*. *J Biochem (Tokyo)* 1994; **116**: 986-990

13 **Davies WD**, Pittard J, Davidson BE. Cloning of *aro*G, the gene coding for phospho-2-keto-3-deoxy-heptonate aldolase (phe), in *Escherichia coli* K-12, and subcloning of the *aro*G promoter and operator in a promoter-detecting plasmid. *Gene* 1985; **33**: 323-331

14 **Davies WD**, Davidson BE. The nucleotide sequence of *aro*G, the gene for 3-deoxy-D-arabinoheptulosonate-7-phosphate synthetase (phe) in *Escherichia coli* K12. *Nucleic Acids Res* 1982; **10**: 4045-4048

15 **Chao YP**, Lai ZJ, Chen P, Chern JT. Enhanced conversion rate of L-phenylalanine by coupling reactions of aminotransferases and phosphoenolpyruvate carboxykinase in *Escherichia coli* K-12. *Biotechnol Prog* 1999; **15**: 453-458

16 **Yang J**, Pittard J. Molecular analysis of the regulatory region of the *Escherichia coli* K-12 *tyr*B gene. *J Bacteriol* 1987; **169**: 4710-4715

17 **Fotheringham IG**, Dacey SA, Taylor PP, Smith TJ, Hunter MG, Finlay ME, Primrose SB, Parker DM, Edwards RM. The cloning and sequence analysis of the *asp*C and *tyr*B genes from *Escherichia coli* K12. Comparison of the primary structures of the aspartate aminotransferase and aromatic aminotransferase of *E. coli* with those of the pig aspartate aminotransferase isoenzymes. *Biochem J* 1986; **234**: 593-604

18 **Zhang S**, Wilson DB, Ganem B. Probing the catalytic mechanism of prephenate dehydratase by site-directed mutagenesis of the *Escherichia coli* P-protein dehydratase domain. *Biochemistry* 2000; **39**: 4722-4728

19 **Nelms J**, Edwards RM, Warwick J, Fotheringham I. Novel mutations in the *phe*A gene of *Escherichia coli* K-12 which result in highly feedback inhibition-resistant variants of chorismate mutase/prephenate dehydratase. *Appl Environ Microbiol* 1992; **58**: 2592-2598

20 **Gavini N**, Davidson BE. *phe*Ao mutants of *Escherichia coli* have a defective *phe*A attenuator. *J Biol Chem* 1990; **265**: 21532-21535

21 **Gowrishankar J**, Pittard J. Regulation of phenylalanine biosynthesis in *Escherichia coli* K-12: control of transcription of the *phe*A operon. *J Bacteriol* 1982; **150**: 1130-1137

22 **Ikeda M**, Ozaki A, Katsumata R. Phenylalanine production by metabolically engineered *Corynebacterium glutamicum* with the *phe*A gene of *Escherichia coli*. *Appl Microbiol Biotechnol* 1993; **39**: 318-323

23 **Oh MK**, Rohlin L, Kao KC, Liao JC. Global expression profiling of acetate-grown *Escherichia coli*. *J Biol Chem* 2002; **277**: 13175-13178

24 **Inui M**, Nakata K, Roh JH, Zahn K, Yukawa H. Molecular and functional characterization of the *Rhodopseudomonas palustris* no. 7 phosphoenolpyruvate carboxykinase gene. *J Bacteriol* 1999; **181**: 2689-2696

25 **Scovill WH**, Schreier HJ, Bayles KW. Identification and characterization of the *pck*A gene from *Staphylococcus aureus*. *J Bacteriol* 1996; **178**: 3362-3364

26 **Osteras M**, Driscoll BT, Finan TM. Molecular and expression analysis of the *Rhizobium meliloti* phosphoenolpyruvate carboxykinase (*pck*A) gene. *J Bacteriol* 1995; **177**: 1452-1460

27 **Pantaik R**, Liao JC. Engineering of *Escherichia coli* central metabolism for aromatic metabolite production with near theoretical yield. *Appl Environ Microbiol* 1994; **60**: 3903-3908

28 **Jiang PH**, Shi M, Qian ZK, Li NJ, Huang WD. Effect of F209S mutation of *Escherichia coli aro*G on resistance to phenylalanine feedback inhibition. *Shengwu Huaxue Yusheng Wuwuxi Xuebao* 2000; **32**: 441-444

29 **Niersbach M**, Kreuzaler F, Geerse RH, Postma PW, Hirsch HJ. Cloning and nucleotide sequence of the *Escherichia coli* K-12 *pps*A gene, encoding PEP synthase. *Mol Gen Genet* 1992; **231**: 332-336
30 **Medina V**, Pontarollo R, Glaeske D, Tabel H, Goldie H. Sequence of the *pck*A gene of *Escherichia coli* K-12: relevance to genetic and allosteric regulation and homology of *E. coli* phosphoenolpyruvate carboxykinase with the enzymes from *Trypanosoma brucei* and *Saccharomyces cerevisiae*. *J Bacteriol* 1990; **172**: 7151-7156
31 **Bradford MM**. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**: 248-254
32 **Hutchins AM**, Holden JF, Adams MW. Phosphoenolpyruvate synthetase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *J Bacteriol* 2001; **183**: 709-715
33 **Laivenieks M**, Vieille C, Zeikus JG. Cloning, sequencing, and overexpression of the *Anaerobiospirillum succiniciproducens* phosphoenolpyruvate carboxykinase (*pck*A) gene. *Appl Environ Microbiol* 1997; **63**: 2273-2280
34 **Xia T**, Zhao G, Jensen RA. Loss of allosteric control but retention of the bifunctional catalytic competence of a fusion protein formed by excision of 260 base pairs from the 3' terminus of pheA from *Erwinia herbicola*. *Appl Environ Microbiol* 1992; **58**: 2792-2798
35 **Zhang S**, Wilson DB, Ganem B. Probing the catalytic mechanism of prephenate dehydratase by site-directed mutagenesis of the *Escherichia coli* P-protein dehydratase domain. *Biochemistry* 2000; **39**: 4722-4728
36 **Baker CS**, Morozov I, Suzuki K, Romeo T, Babitzke P. CsrA regulates glycogen biosynthesis by preventing translation of *glg*C in *Escherichia coli*. *Mol Microbio* 2002; **44**: 1599-1610
37 **Gudapaty S**, Suzuki K, Wang X, Babitzke P, Romeo T. Regulatory interactions of Csr components: the RNA binding protein CsrA activates *csr*B transcription in *Escherichia coli*. *J Bacteriol* 2001; **183**: 6017-6027
38 **Nogueira T**, Springer M. Post-transcriptional control by global regulators of gene expression in bacteria. *Curr Opin Microbiol* 2000; **3**: 154-158
39 **Wei B**, Shin S, LaPorte D, Wolfe AJ, Romeo T. Global regulatory mutations in *csr*A and *rpo*S cause severe central carbon stress in *Escherichia coli* in the presence of acetate. *J Bacteriol* 2000; **182**: 1632-1640
40 **Romeo T**. Global regulation by the small RNA-binding protein CsrA and the non-coding RNA molecule CsrB. *Mol Microbiol* 1998; **29**: 1321-1330
41 **Liu MY**, Romeo T. The global regulator CsrA of Escherichia coli is a specific mRNA-binding protein. *J Bacteriol* 1997; **179**: 4639-4642

Edited by Ren SY