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Construction of Plasmids for Overproduction of L-Phenylalanine

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L-페닐알라닌 대량생산을 위한 재조합 플라스미드 구성

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ABSTRACT: For the overproduction of L-phenylalanine using *Escherichia coli*, the authors constructed various recombinant plasmids including pMW 10, pMW 11 and pMW 12. The *aroF*^{FR} and *pheA*^{FR} genes for the production of L-phenylalanine were isolated from *Escherichia coli* MWEC 101-5 strains. The productivity and stability of *Escherichia coli* regulatory mutants containing recombinant plasmids were investigated to evaluate the efficiency of the *aroF*^{FR} and *pheA*^{FR} genes. The MWEC 101-5/pMW 11 strain produced 24.3 g/l of L-phenylalanine while its stability was 73.8 percent. The specific activity of prephenate dehydratase in the MWEC 101-5/pMW 11 strain increased by 26-fold compared with that of *Escherichia coli* K-12.

KEY WORDS L-Phenylalanine, recombinant plasmid, enzyme activity

L-Phenylalanine is the raw material for L-α-aspartyl-L-phenylalanine methylester, a new peptide sweetner (Klausner, 1985). A growing demand for L-phenylalanine has promoted the industrial application of microbial processes, including direct fermentation from glucose or other conventional carbon sources, microbial conversion of biosynthetic intermediates and enzymatic synthesis (Tsuchida *et al.*, 1975; Choi and Tribe, 1982; Calton *et al.*, 1986; Yamada *et al.*, 1981; Marusich *et al.*, 1981). The production of L-phenylalanine by fermentation methods has the disadvantages of high capital costs, high labor costs, instability of producing strains and relatively high substrate costs. The application of recombinant DNA techniques to microorganisms should improve significantly the economy of the fermentative processes.

The L-phenylalanine biosynthesis is mainly controlled at two reaction steps by the feedback inhibition and the repression of end products, such as phenylalanine and tyrosine. One of the regulated steps is the conversion of D-erythrose-4-phosphate and phosphoenolpyruvate into 3-deoxy-D-arabino-heptulosonate-7-phosphate by three isoenzymes of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase(DAHP synthase) encoded on *aroF*, *aroG* and *aroH* genes (McCandliss *et al.*, 1978; Le Marechal *et al.*, 1980; Pittard *et al.*, 1969). The other is the conversion of chorismic acid to phenylpyruvate by chorismate mutase and prephenate dehydratase encoded on *pheA* gene (Davidson *et al.*, 1972).

The main focus of this study was the construction of various recombinant plasmids for the overproduction of L-phenylalanine by *E. coli* involving the cloning of the mutated gens *aroF*^{FR} (FR: feedback inhibition resistance) and *pheA*^{FR} that encoded DAHP synthase, chorismate mutase and prephenate dehydratase.

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Table 1. *Strains and plasmids used in this study*

Strains and Plasmids	Relevant properties	Reference or source
E. coli K-12	wild type	our collection
MWEC 101-5	PAP^r, 3-AT^r, β-TA^r, Val^r	our laboratory
MWEC 101-6-7	PAP^r, MFP^r, β-TA^r, Val^r	〃
MWEC 203-7	*pheA* deficiency strain	〃
Plasmid pBR 322	Amp^r, Tc^r	F. Bolivar *et al.*
pMK 20	Km^r	our collection
pPLc 2833	Amp^r	E. Remaut *et al.*
pMW 10	pBR 322-*pheA*^{FR}*aroF*^{FR}	in this study
pMW 11	pMK 20-pMW 10 hybrid	〃
pMW 12	pPLc 2833-pMW 11 hybrid	〃

pheA^{FR} and *aroF*^{FR} indicate that the enzyme encoded by *pheA* and *aroF* genes are desensitized to end product inhibition.

MATERIALS AND METHODS

Strains and plasmids

All strains were derived from *E. coli* K-12. The strains and plasmids used in this study are presented in Table 1. Strain MWEC 203-7, which is deficient in chorismate mutase and prephenate dehydratase, was used as a host strain for selection of the plasmids carrying *pheA*$^+$ genes.

Medium, enzymes and chemicals

Luria broth (LB) used for complete medium. The minimal medium (MM) was used as a selective medium in genetic experiments and growth of cells for cell extract preparation. The fermentation media, pH 7.0, contained per liter 50g of glucose, 20g of $(NH_4)_2SO_4$, 1g of KH_2PO_4, 1g of K_2HPO_4, 1g of K_2HPO_4, 1g of K_2SO_4, 10mg of $FeSO_4 \cdot 7H_2O$, 10mg of $MnSO_4 \cdot 7H_2O$, 5mg of $CoCl_2 \cdot 6H_2O$, 10mg of $ZnSO_4 \cdot 7H_2O$, 2g of $MgSO_4 \cdot 7H_2O$, 5mg of thiamine HCl and 5mg of nicotinic acid. Cells were grown in a 5-liter fermenter (model F-2000; New Brunswick Scientific Co., New Brunswick, U.S.A.). Restriction endonucleases were purchased from Bethesda Research Laboratories (BRL) or New England Biolabs (NEB). T_4DNA ligase and T_4DNA polymerase were obtained from BRL and Promega Biotec, respectively. Most of substances for electrophoresis were obtained from Sigma Chemical Co. All other chemicals were of reagent grade. All enzymes and buffers were used as recommended by the manufacturers.

Construction of recombinant plasmids

A large-scale isolation for plasmid DNA was performed by the method of Cold Spring Harbor Laboratory Manual (Maniatis *et al.*, 1982). Covalent closed circular DNA molecules were purified by the CsCl-ethidium bromide equilibrium density gradient centrifugation. The digestion of plasmid DNA was performed according to the methods described by Maniatis *et al.*, (1982). The digested DNA fragments were purified by electrophoresis on agarose gels. Ligation was performed overnight at 15°C in 20 mM Tris (hydroxymethyl) aminomethane-hydrochloride buffer (pH 8.0) containing 10 mM $MgCl_2$, 5 mM dithiothreitol and 0.5 mM ATP (Weiss *et al.*, 1968).

Transformation of *E. coli*

Cells of *E. coli* regulatory mutants were transformed using the method of calcium chloride as presented by Nordgard (1978). The transformed cells were grown on LB agar plates containing required antibiotics.

Stability of recombinant plasmids

For the study of recombinant plasmid stability, cells grown in minimal medium or production medium of phenylalanine were spreaded on LB agar plate, and then the colonies grown on LB agar plate were toothpicked on minimal medium or selection medium containing antibiotics.

Preparation of crude extracts and enzyme assay

After separation by centrifuge, cells were washed in ice cold 0.9% (w/v) NaCl solution and suspended in 5 ml of appropriate buffers (Choi, 1981). Cell breakage was then achieved using a Fisher sonic dismembrater (Fisher, Model 300, West Germany), for a total 2 to 4 minutes. Extracts were clarified using a centrifuge, then the supernatant was purified by $(NH_4)_2SO_4$ precipitation. The activities of chorismate mutase, prephenate dehydratase and DAHP synthase were assayed by the methods of Gething *et al.*, (1976), Cotton and Gibson (1970) and Camakaris (1971).

RESULTS AND DISCUSSION

Construction of recombinant plasmids pMW 10, pMW 11 and pMW 12

The *aroF*^{FR} (FR: feedback-inhibition resistant) and *pheA*^{FR} gene for L-phenylalanine production were derived from *E. coli* MWEC 101-5 by a shotgun method. At this time, the *E. coli* MWEC 101-5 was free from the feedback inhibition by end products.

After MWEC 101-5 strain was cultivated in LB media at 37 °C for 15 hrs, the cells were harvested. The chromosomal DNA (cDNA) was isolated by the alkaline extraction method and CsCl density gradient centrifuge method. Thereafter, the cDNA was purified by butanol treatment and dialysis. Plasmids from *E. coli* HB 101/pBR 322 and *E. coli* HB 101/pMK 20 were also purified. The cDNA was digested with Eco RI in medium salt restriction enzyme buffer (50 mM of sodium chloride, 10 mM of Tris (pH 7.5), 10 mM of magnesium chloride, and 1 mM of dithiothreitol) at 37 °C for one hour. The restriction enzyme was inactivated in a conventional manner, and the cDNA was further digested with the restriction enzyme Pst I.

A 4-7 kb gene fragment was recovered from 0.7% (w/w) agarose gel to be a target gene. The plasmid pBR 322 was also digested by the restriction enzyme Eco RI and Pst I. The digested plasmid pBR 322 was mixed with the 4-7 kb gene fragment produced from *E. coli* MWEC 101-5. The digested plasmid and gene fragment were combined with each other in T_4DNA ligase buffer solution (0.5M of Tris (pH 7.4), 0.1M of magnesium chloride, 0.1M of dithiothreitol, 10 mM of spermidine, 10 mM of ATP, 1 mg/ml of bovine serum albumin) at 12-14 °C for 12 hours. The ligation mixture was transformed into MWEC 203-7 to obtain plasmid pMW 10 by $CaCl_2$ method (Nordgard, 1978). The transformed strains were selected on LB agar plate containing 15 ug/ml of tetracycline. The plasmid pMW 10 was isolated from transformant and digested with Hind III restriction enzyme, partially digested again with Pst I restriction enzyme. 4.2 kb Hind III-Pst I fragment containing *pheA* and *aroF* gene was separated from 0.7% agarose gel. T_4DNA polymerase and dNTP mixture solution (25 mM of dATP, 25 mM of dGTP, 25 mM of dCTP and 25 mM of dTTP) were added to the plasmid to convert the sticky ends to blunt ends.

Plasmid pMK 20 containing kanamycin resistance (km^r) gene was treated with Pst I and convert its sticky ends to blunt ends. According to the same method as mentioned above, the recombinant plasmid pMW 11 was isolated from transformants which grew on MM agar plate containing 50 μg/ml of kanamycin. The procedure of plasmid construction of pMW 10 and pMW 11 are shown in Fig. 1.

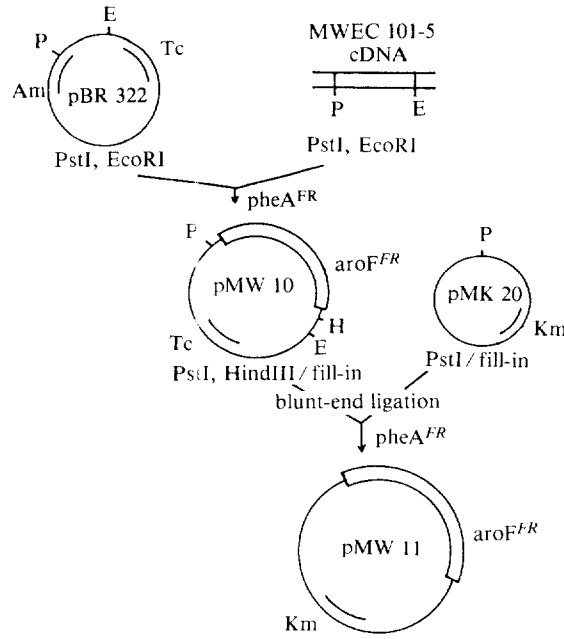

Fig. 1. *Diagramatic representation of the construction of recombinant plasmid pMW 10 and pMW 11.*

The P_L promoter of bacteriophage is a strong, well-regulated promoter that has been used in several expression vectors. To further the expression of cloned genes, P_L promoter was introduced by pPLc 2833 plasmid. The plasmid pPLc 2833 which contains the P_L promoter was treated with Bam HI and Hae II restriction enzyme and a 0.2 kb P_L fragment was recovered on 0.2% agarose gel. The fragment was treated with T_4DNA polymerase and dNTP to form blunt ends at both ends. The recombinant plasmid pMW 11 was digested with Afl II and treated by the same method as the above-mentioned to form blunt ends at both ends. The P_L fragment was added to the treated pMW 11 and ligated to give a plasmid pMW 12. The overall scheme of plasmid construction is depicted in Fig. 2.

L-phenylalanine production and phenotypic stability of plasmid-harboring *E. coli* regulatory mutants

The various *E. coli* regulatory mutants were transformed with the recombinant plasmids to produce a novel strain for use in manufacturing the L-phenylalanine. The L-phenylalanine production by the transformants was tested to evaluate the effectiveness of the *aroF*$^+$ and *pheA*$^+$ genes. The biochemical pro-

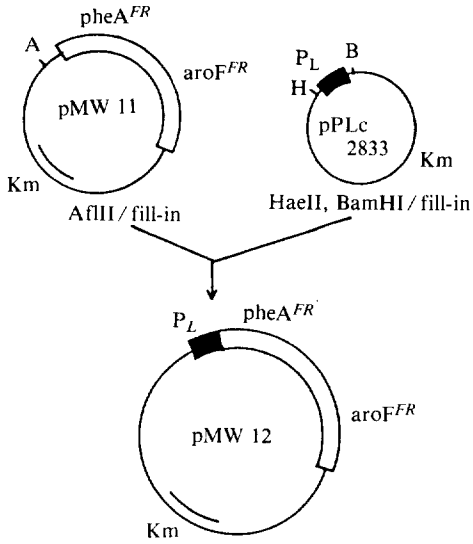

Fig. 2. *Diagramatic representation of the construction of recombinant plasmid pMW 12.*

Table 2. *L-Phenylalanine production and phenotypic stability of E. coli regulatory mutants containing recombinant plasmids*

Strain/Plasmid	L-Phenylalanine produced(g/l)[a]	Plasmid Stability(%)[b]
MWEC 101-5	17.9	—
MWEC 101-5/pMW 10	19.6	57.2
MWEC 101-5/pMW 11	24.3	73.8
MWEC 101-5/pMW 12	22.7	65.2
MWEC 101-6-7	16.4	—
MWEC 101-6-7/pMW 10	18.9	51.3
MWEC 101-6-7/pMW 11	23.5	78.7
MWEC 101-6-7/pMW 12	21.2	76.8

a. Production of L-phenylalanine was performed in a 5-liter jar fermenter.

b. The number of colonies carrying the plasmid/the total number of colonies checked.

perties of the transformants were the same as those of host strains (unpublished results). However, the yield of L-phenylalanine of the transformants increased significantly. As shown in Table 2, recombinant plasmid pMW 11 harboring *E. coli* strains produced a large amount of L-phenylalanine. When pMW 11 was introduced into MWEC 101-5, the amount of L-phenylalanine produced increased from 17.9 g/l to 24.3 g/l in a 5-liter jar fermenter. This increase in production seemed to be due the gene dosage effect on the production of specific enzymes.

Table 3. *Chorismate mutase and prephenate dehydratase activities of E. coli mutants carrying recombinant plasmids pMW 10, pMW 11 and pMW 12*

Strain/Plasmid	Specific activity[a] (Relative activity)	
	CMase	PDase[b]
E. coli K-12	1.30(1.0)	0.71(1.0)
MWEC 101-5	12.81(9.9)	14.6 (20.6)
MWEC 101-5/pMW 11	15.0 (11.5)	18.3 (25.7)
MWEC 101-5/pMW 12	14.8 (11.4)	16.5 (23.2)
MWEC 101-6-7	11.73(9.0)	11.1 (15.6)
MWEC 101-6-7/pMW 10	12.3 (9.5)	16.7 (23.5)
MWEC 101-6-7/pMW 11	15.6 (12.0)	17.3 (24.3)
MWEC 101-6-7/pMW 12	13.0 (10.0)	16.1 (22.7)

a. Specific activities are given as units of enzyme activity per mg protein.

b. Abbreviations: CMase, chorismate mutase; PDase, prephenate dehydratase.

Table 4. *DAHP synthase activity of recombinant plasmid harboring E. coli*

Strain/Plasmid	Cell growth	Specific activity
MWEC 101-5	0.22	14.7
MWEC 101-5/pMW 11	0.27	16.9
MWEC 101-6-7	0.25	12.0
MWEC 101-6-7/pMW 12	0.26	16.3

Enzyme assay was carried out as described under Materials and Methods. Specific activity is given as units of enzyme activity per mg of protein.

Although these transformants produced L-phenylalanine in abundance, their stability was unsatisfactory in the commercial production. Efforts were made in this laboratory to improve the stability of the recombinant plasmids and to further enhance the productivity.

Enzyme activities of recombinant plasmid-harboring *E. coli* regulatory mutants

Chorismate mutase, prephenate dehydratase and DAHP synthase were assayed in the transformants. As shown in the Table 3, prephenate dehydratase activity of transformants was higher than that of the choriamate mutase. Especially, the specific activity of prephenate dehydratase in the transformant containing pMW 11 increased more than 24-fold to 26-fold compared with that of *E. coli* K-12. When the recombinant plasmids were transformed, an increase

in the enzyme activity varied on the hosts. Meanwhile introducing pMW 11 into the MWEC 105-5 a 25 percent increase in prephenate dehydratase activity was observed, but in the MWEC 101-6-7 containing pMW 11, it increased by 56 percent.

These results suggest that the development of host is very important. It is also thought that chorismate mutase activity, besides prephenate dehydratase activity, should be intensified to increased production of L-phenylalanine. Table 4 shows DAHP synthase activities of the mutants obtained in this study. DAHP synthase was over-produced by an approximate by 40 percent as a result of introducing pMW 11 into MWEC 101-5. In the introduction of recombinant plasmids, the enzymes related to L-phenylalanine specific pathway seem to contribute to L-phenylalanine biosynthesis more than DAHP synthase does.

적 요

*E. coli*를 이용하여 L-phenylalanine을 대량 생산하기 위한 재조합 플라스미드 pMW 10, pMW 11과 pMW 12를 구성하였다. L-Phenylalanine 생산을 위한 유전자 $aroF^{FR}$, $pheA^{FR}$은 *E. coli* MWEC 101-5 균주로부터 분리하였다. 재조합 플라스미드를 함유하고 있는 *E. coli* 대사 조절변이주들의 L-phenylalanine 생산성과 안전성을 조사하여 $aroF^{FR}$, $pheA^{FR}$ 유전자들의 효율을 알아보았다. MWEC 101-5/pMW 11 균주에서는 24.3 g/*l*의 L-phenylalanine이 생산되었으나, 플라스미드의 안정성은 73.8%였다. 본 균주의 prephenate dehydratase 고유 활동도는 *E. coli* K-12에 비하여 26배 증가된 것이었다.

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