L-Phenylalanine Production by Regulatory Mutants of *Escherichia coli* K-12

Lee*, Sae-Bae, Chung Park, Chan-Hee Won, Duk-Ho Choi and Bun-Sam Lim

R & D Center, Miwon Co., Ltd., 720, Banghak-dong Dobong-ku, Seoul 132, Korea

Escherichia coli K-12 대사조절 변이주에 의한 L-페닐알라닌 생산

이새배 · 박 청 · 원찬희 · 최덕호 · 임번삼

(주)미원 중앙연구소

ABSTRACT: In order to overproduce L-phenylalanine, various kind of regulatory mutants were isolated from parental *Escherichia coli* K-12. MWEC 83 Producing 7.4 g/l of L-phenylalanine has been derived as a tyrosine and tryptophan double auxotrophic mutant. To produce L-phenylalanine without adding L-tyrosine and L-tryptophan, revertant strain MWEC 101 was isolated from MWEC 83. Further various analogues and valine resistant mutants were isolated from MWEC 101. MWEC 101-5 was the most excellent strain that produced 17.9 g/l of L-phenylalanine after having been cultivated for 54 hours in 15% glucose medium. It was disclosed that activities of rate-limiting enzymes including chorismate mutase and prephenate dehydratase in MWEC 101-5 were desensitized to 2mM L-phenylalanine in the enzyme reaction mixture and that activities level of 3-deoxy-D-arabino-heptulosonic acid-7-phosphate synthase and prephenate dehydratase were increased more than 20 times over those of the parental strain.

KEY WORDS L-phenylalanine, regulatory mutant, enzyme activity

L-Phenylalanine is an important amino acid not only as an essential amino acid for human nutrition, but also as a component for use in medical infusions. Further, it is now in use as an important raw material of the diet sweetner aspartame, which is a peptide of L-phenylalanine methyl ester and L-aspartic acid. Thus the demand for L-phenylalanine is on a rapid increase, along with L-aspartic acid.

A variety of methods have been used for the production of L-phenylalanine. These include extraction from natural proteins, chemical synthesis, production from trans-cinnamic acid (Hattori *et al.*, 1981; Yamada *et al.*, 1981) and the direct fermentation of sugar by *Brevibacterium* sp., *Corynebacterium* sp. (Nakayama *et al.*, 1973; Hagino *et al.*, 1974) and *E. coli* (Choi and Tribe, 1982).

Im and Pittard (1971) isolated mutants of *E. coli* resistant to *o*- and *p*-fluoro-phenylalanine which were constitutively depressed by chorismate mutase (CMase) and prephenate dehydratase (PDase). Choi and Tribe (1982) obtained L-phenylalanine overproducing strains of *E. coli* by the "systematic construction of multiple mutation strains" in which metabolic regulation was released. This strain produced 8.7 g/l of L-phenylalanine. Hwang *et al.* isolated a tyrosine auxotroph which was resistant to PFP (*p*-fluorophenylalanine) and β-TA (β-(2-thienyl)DL-alanine) that overproduced L-phenylalanine. These mutants accumulated 15 g/l of L-phenylalanine. Gil *et al.* (1985) used these mutants in a pilot plant to produce 15 g/l of L-phenylalanine.

In this report the authors described the isolation and properties of *E. coli* regulatory mutants which overproduced L-phenylalanine. The mutant numbered MWEC 101-5 was found to be the best for L-phenylalanine production. This strain produced 17.9 g/l of L-phenylalanine and had genetically desensitizied DAHP synthase (DAHPase) and chorismate mutase

*To whom correspondence should be addressed

(CMase)-prephenate dehydratase (PDase).

MATERIALS AND METHODS

Strains and media

All mutants were directly derived from *E. coli* K-12. The minimal medium (MM) was used as a selective medium in the isolation of regulatory mutants and for the growth of cells in cell extract preparation. The MM medium, pH 7.0, contained the following components per liter: 5g of glucose, 1g of $(NH_4)_2SO_4$, 2g of KH_2PO_4, 7g of K_2HPO_4, 0.1g of $MgSO_4 \cdot 7H_2O$ and 0.5g of Na-citrate. The fermentation medium, 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_2SO_4, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 7H_2O$, 5 mg of $CoCl_2 \cdot 6H_2O$, 10 mg of $ZnSO_4 \cdot 7H_2O$, 0.2g of $MgSO_4 \cdot 7H_2O$, 5 mg of thiamine HCl and 5 mg of nicotinic acid. The pH was controlled by the automatic addition of 1N NH_4OH. Cells were grown in a 5-liter fermenter (Model F-2000; New Brunswick Scientific Co., U.S.A.), operated with a liquid volume of 3 liters.

Isolation of mutants

Cells were mutagenized by the treatment with N-methyl-N'-nitro-N-nitrosoguanidine and/or ultraviolet light irradiation. Ampicillin was used for antibiotic enrichment of amino acid auxotrophs.

Cell growth

The growth of cells was monitored by the spectrophotometer at the optical density of 610 nm.

Chemicals

Phosphoenolpyruvate, erythrose-4-phosphate, Ba-prephenate, Ba-chorismate, aromatic amino acid analogues were purchased from Sigma Chemical Co. All other chemicals used in the experiment were of analytical reagent grade.

Preparation of cell extracts

After centrifugal separation at 7,000 rpm for 10 minutes at 4°C, cells were washed twice in ice-cold 0.9% (w/v) NaCl solution and suspended in 5 m*l* of appropriate buffers (Choi, 1981). Cell breakage was then achieved using a Fisher sonic dismembrator (Fisher, Model 300, West Germany), for two to four minutes. Between bursts of 30 seconds, the cell suspensions were chilled in an ice-bath for 60 seconds to minimize enzyme denaturation from overheating. Extracts were clarified by centrifugal separation at 12,000 rpm for 20 minutes and then assayed immediately.

Determination of enzyme activity

DAHP synthase (EC.4.1.2.15) activity was determined by measuring the amount of DAHP formed from erythrose-4-phosphate and phosphoenolpyruvate (Camakaris, 1971; Srinivasan and Sprinson, 1959). The incubation mixture contained 0.58 μmole of erythrose-5-phosphate, 0.5 μmole of phosphoenolpyruvate, and a rate limiting amount of enzyme in a total volume of 0.5 m*l*. DAHP was estimated according to the method of Doy and Brown (1965). The concentration of DAHP and hence the specific activity was calculated using the molar extinction coefficient of 33,400.

Chorismate mutase and prephenate dehydratase assays were carried out as described by Gething *et al.* (1976). Chorismate mutase was assayed by measuring the rate of conversion from chorismate to prephenate and phenylpyruvate. The activity was assayed in a 0.4 m*l* reaction mixture containing 1 mM chorismate, 50 mM Tris-Cl, pH 7.8, 2.5 mM EDTA, 0.01% bovine serum albumin and 20 mM 2-mercaptoethanol. Prephenate dehydratase was assayed by measuring the rate of conversion from prephenate to phenylpyruvate. The activity was assayed in a 0.4 m*l* reaction mixture containing 0.5 mM barium prephenate, 20 mM Tris-Cl, pH 8.2, 1.0 mM EDTA, 0.01% bovine serum albumin and 20 mM 2-mercaptoethanol.

Protein determination

Protein determination of the cell extracts was carried out colorimetrically using the method of Lowry *et al.*, (1951) with bovine serum albumin as a standard.

Unit of enzyme activity

Enzyme assays were all carried out at 30°C under conditions of linearity with respect to incubation time and the amount of enzyme used. The unit of activity is the amount of enzyme converting 1 μmole of product per minute at 30°C. Specific activities are given as a unit of enzyme activity per mg of protein.

L-Phenylalanine analysis

L-Phenylalanine was assayed by HPLC using a Waters C 18 column with a mobile phase of 35% methanol, 0.05M phosphate buffer, pH 7.2, and also using and OPA (orthophtaldehyde) precolumn derivatization.

RESULTS AND DISCUSSION

Isolation of regulatory mutants

In order to isolate regulatory mutant, *E. coli* K-12 grown on a minimal medium were centrifuged and resuspended in an equal volume of saline. As a survival of 0.1-1% is used for mutagenesis.

(a) Auxotroph isolation

Table 1. *L-Phenylalanine production by some of the auxotrophic mutants derived from E. coli K-12*

Strains	Phenotype[a]	L-Phenylalanine produced (mg/ml)[b]
E. coli K-12	wild type	—
MWEC 23	Tyr^-	2.3
MWEC 24	Tyr^-	3.8
MWEC 27	Tyr^{lea}	5.4
MWEC 36	Tyr^{lea}, Trp^-	6.0
MWEC 83	Tyr^-, Trp^-	7.4
MWEC 87	Trp^-	3.1
MWEC 90	Tyr^{lea}, Trp^-	5.7

[a]Abbreviation: Tyr^-, Tyrosine auxotroph; Tyr^{lea}, Tyrosine leaky; Trp^-, Tryptophan auxotroph; Trp^{lea}, Tryptophan leaky.

[b]Fermentation was carried out in a 5-liter jar fermenter containing the production medium supplemented with 20 mg/ml of required amino acids.

L-Phenylalanine production by various tyrosine and/or tryptophan auxotroph is summarized in Table 1. The best strain MWEC 83 produced 7.4 g/*l* of L-phenylalanine which was a tyrosine, tryptophan double auxotrophic mutant.

(b) Analogue resistant mutant

In order to improve the yield of L-phenylalanine, the authors tried to isolate various analogue resistant mutants from the *E. coli* auxotrophic mutant previously mentioned in Table 1. The isolation of mutants in which the mutation desensitizes to phenylalanine inhibition was also achieved by the use of the phenylalanine analogues. In Fig. 1 the degree of growth inhibition for *E. coli* K-12 by various L-phenylalanine analogues is shown. From the above results, PAP, β-TA, MFP and 3-AT were choosen as analogues for the isolation of various analogues resistant mutant for exertion of a strong inhibition on growth.

In Table 2 various phenylalanine and/or tyrosine analogue resistants obtained in these experiments are summarized, together with the level of L-phenylalanine. The strain MWEC 83-101 produced 11.3 g/*l* of L-phenylalanine which was a PAP (5 mg/ml) resistant mutant. In order to produce L-phenylalanine without the addition of tyrosine and tryptophan, strain MWEC 101 isolated from MWEC 83, tyrosine and tryptophan double auxotroph. Almost all strains of revertants produced relatively small amounts of phenylalanine. Also, the authors isolated various analogues and valine resistant mutant from MWEC

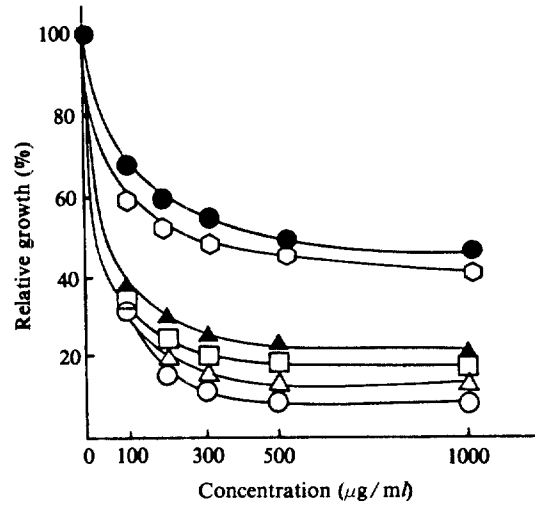

Fig. 1. *Degree of growth inhibition for E. coli K-12 by various phenylalanine and tyrosine analogues.* Symbols used: (○), p-amino-DL-phenylalanine; (△), β-(2-Thienyl)DL-alanine; (□), m-fluoro-DL-phenylalanine; (○), o-fluoro-DL-phenylalanine; (●), p-fluoro-DL-phenylalanine and (▲), 3-amino tyrosine.

Table 2. *L-Phenylalanine production by some of E. coli regulatory mutants derived from auxotrophic mutants*

Strains	Characteristics	L-Phenylalanine produced (g/l)[a]
MWEC 23-54	Tyr^-, PAP^r, MFP^r	6.6
MWEC 27-36	Tyr^{lea}, MFP^r, β-TA^r	8.7
MWEC 101-3	PAP^r, 3-AT^r, β-TA^r	15.1
MWEC 101-5	PAP^r, 3-AT^r, β-TA^r, Val^r	17.9
MWEC 101-6-7	PAP^r, MFP^r, β-TA^r, Val^r	16.4
MWEC 83-101	Tyr^-, Trp^-, PAP^r	11.3
MWEC 90-54	Tyr^{lea}, Trp^{lea}, PAP^r	9.6

[a]The cultivation was carried out at 30°C for 50 hrs in fermentation medium.

101. The best strain MWEC 101-5 produced 17.9 g/*l* of L-phenylalanine. Especially, valine exerted a great degree of inhibition on growth at 3 mg/ml concentration in *E. coli* (unpublished results). Also, it may be that the valine resistance in improved L-phenylalanine producers contributes to increasing the yield of L-phenylalanine.

Enzyme analysis of *E. coli* regulatory mutants

Chorismate mutase, prephenate dehydratase and DAHP synthase were assayed in the crude enzyme

preparation from various regulatory mutants. As shown in Table 3, in the best strain MWEC 101-5, both DAHP synthase and prephenate dehydratase activity increased over twenty times higher than those of the parent strain *E. coli* K-12. These results indicated that the prephenate dehydratase activity in phenylalanine overproducers was more important than chorismate mutase for L-phenylalanine production. Both activities of the CMP-PDH complex, namely chorismate mutase and prephenate dehydratase activities are reported to be feedback controlled by the allosteric inhibitor phenylalanine, although the dehydratase activity is generally more sensitive than the mutase activity (Dopheide *et al.*, 1972; Gething *et al.*, 1976). Data illustrating the degree to which chorismate mutase and prephenate dehydratase of the strain MWEC 101-5 differ from those of the reference strains, *E. coli* K-12, MWEC 101-3 and MWEC 101-6-7 in the sensitivity to feedback inhibition are given in Table 4. From the results shown in the Table 4, the control strains *E. coli* K-12 show 90-92 percent inhibition of chorismate mutase and prephenate dehydratase activity in the presence of 2 mM phenylalanine, while on the other strains, both enzyme activities were approximately desensitized to 2 mM phenylalanine. Also these show that the prephenate dehydratase activity in these improved L-phenylalanine producers is more desensitized to phenylalanine inhibition. It may be one of the factors that led to the overproduction of L-phenylalanine in this experiments. However, it is not clear why the activity of prephenate dehydratase is more desensitized.

It has been reported that the synthesis of the three 3-deoxy-arabino-heptulosonate-7-phosphate(DAHP)

Table 3. *Enzyme activities of E. coli regulatory mutants*

Strains	Specific activities (relative activities)		
	CMase	PDase	DAHPase
E. coli K-12	1.3(1.0)	0.7(1.0)	0.68(1.0)
MWEC 27-36	8.9(6.9)	10.3(14.5)	7.6(11.2)
MWEC 101-3	10.0(7.7)	10.9(15.3)	8.1(11.9)
MWEC 101-5	12.8(9.9)	14.6(20.6)	14.7(21.6)
MWEC 101-6-7	11.7(9.0)	11.1(15.6)	12.0(17.6)
MWEC 83-101	9.8(7.5)	10.3(14.5)	8.0(11.8)

Enzyme assays were carried out as described under Materials and Methods. Specific activities are given as units of enzyme activity per mg of protein.

Abbreviations: CMase, Chorismate mutase; PDase, Prephenate dehydratase; DAHPase, DAHP synthase.

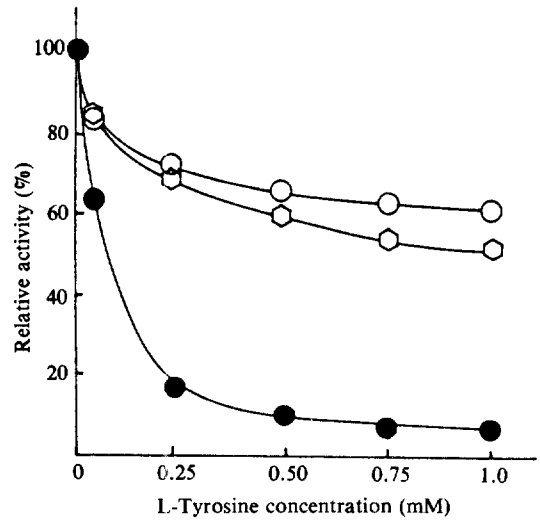

Fig. 2. *Feedback inhibition of DAHP synthase(tyr) by tyrosine.*

Symbols used: (●), *E. coli* K-12; (○), MWEC 101-5 and (O), MWEC 101-6-7.

Table 4. *Sensitivity of chorismate mutase and prephenate dehydratase to L-phenylalanine inhibition for E. coli regulatory mutants*

Strains	Chorismate mutase						Prephenate dehydratase					
	Specific activity	Relative activity in the presence of L-phe(%)					Specific activity	Relative activity in the presence of L-phe(%)				
		0.0	0.25	0.5	1.0	2.0(mM)		0.0	0.25	0.5	1.0	2.0(mM)
E. coli K-12	1.30	100	46.2	31.8	11.7	9.8	0.71	100	38.3	21.6	10.1	7.8
MWEC 101-3	10.0	100	78.3	75.0	70.4	68.3	10.9	100	83.7	80.2	78.6	75.3
MWEC 101-5	12.8	100	92.5	90.8	90.7	90.8	14.6	100	95.2	93.0	92.7	92.5
MWEC 101-6-7	11.7	100	91.7	90.0	89.7	87.4	11.1	100	93.8	92.0	89.9	88.8

[a]Cell extracts were prepared from the cells grown in medium MM at 30°C.

[b]These show the final concentration of L-phenylalanine in the reaction mixture for enzyme assay.

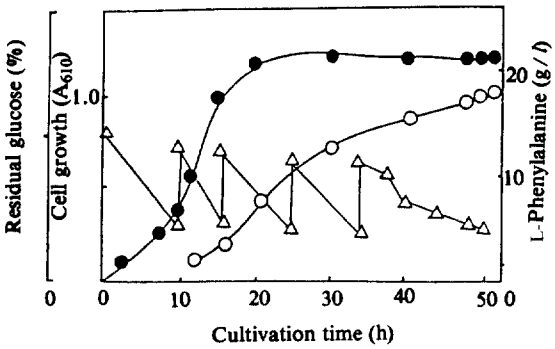

Fig. 3. *Time course of L-phenylalanine production by MWEC 101-5 at 30°C with 5 liter jar fermentor.* Symbols used: (●), Cell growth; (○), L-Phenylalanine and (△), Glucose

synthase isoenzyme are repressible by the corresponding end-product (Tribe and Pittard, 1979; Brown and Somerville, 1971).

But the restriction imposed on phenylalanine overproduction with *E. coli* by these inhibition effects can be easily removed since regulatory mutants of *E. coli* have been isolated in which the sensitivity of each of the DAHP synthases to feedback inhibition was lost (Camakaris and Pittard, 1971). In Fig. 2 the feedback inhibition of DAHP synthase by tyrosine is shown. Control strains show 95 percent inhibition of activity in the presence of 1 mM phenylalanine. The improved strains were desensitized to tyrosine inhibition. Obviously these factors affecting the supply of chorismate are key factors to consider in phenylalanine overproducing strains.

Time course of L-Phenylalanine production

An example of the time course of fermentation by MWEC 101-5 is represented in Fig. 3. From 15% glucose, 17.9 g/*l* of L-phenylalanine was finally obtained after having been cultivated for 54 hours. Determination of the optimum concentration of glucose in fermentation medium is extremely important for the establishment of an economical medium. Strains are under way to define optimum fermentation medium.

적 요

L-Phenylalanine을 대량생산하는 균주를 얻기 위하여 *Escherichia coli* K-12로부터 여러 대사조절 변이주를 분리하였다. MWEC 83은 L-phenylalanine을 7.4 g/*l* 생산하는 tyrosine, tryptophan 이중 영양요구성 변이주이다. Tyrosine과 tryptophan의 첨가없이 L-phenylalanine을 생산하기 위하여 MWEC 83으로부터 복기변이주 MWEC 101을 분리하였다. 또한 MWEC 101 균주로부터 여러 analog와 valine 내성주를 분리하였다. MWEC 101-5는 포도당 15%로 배양 54시간에 17.9 g/*l*의 L-phenylalanine을 생산하는 최고 우량균주이다. MWEC 101-5의 chorismate mutase와 prephenate dehydratase 효소활성은, 효소반응 혼합액 속에 2 mM phenylalanine에 대하여 효소활성이 저해되지 않았다.

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