L-PHENYLALANINE PRODUCTION BY AUXOTROPHIC REGULATORY MUTANTS OF *ESCHERICHIA COLI* — L-Phenylalanine Production by Mutants of *E. COLI*

S.H. Park, K.T. Hong, S.J. You, J.H. Lee* and J.C. Bae

Foods R&D Center, Cheil Sugar & Co., Ltd.
92 Gayang-Dong, Kangseo-Ku, Seoul 150-01, Korea

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Abstract— Various regulatory mutants of *Escherichia coli* have been isolated using phenylalanine and tyrosine analogues. It has been found that the growth of wild type strain of *E. coli* W3110 was strongly inhibited by phenylalanine analogues. Regulatory mutants resistant to phenylalanine analogue could accumulate L-phenylalanine at concentrations of 5-6 g/l. However, L-phenylalanine accumulation was increased significantly up to 11.4 g/l using a tyrosine auxotrophic mutant resistant to phenylalanine analogue such as β-2-thienyl-DL-alanine.

INTRODUCTION

L-phenylalaine is one of the essential raw materials in the manufacture of new sweetener known as "Aspartame". The sweetening potency of aspartame has been assessed to be 150 to 200 times sucrose[1,2]. The market for aspartame is growing steadily around the world and currently commercial L-phenylalanine fermentation is carried out by Japanese amino acid producers such as Kyowa Hakko, Ajinomoto, and Tanabe.

L-phenylalanine fermentation has been studied by several investigators. Tokoro *et al.*[3] reported microbial production of L-phenylalanine from n-alkanes L-phenylalanine concentration up to 10 g/l was accumulated in the fermentation broth. Similar results were obtained using an auxotrophic regulatory mutant of *C. glutamicum* [4]. However, the phenylalanine productivity of 0.15 g/l/h was low and byproducts such as glutamic acid, valine, and alanine were accumulated as the fermentation progressed. Atashi *et al.*[5] used a mutant derived from *B. lactofermentum* and demonstrated the importance of dissolved oxygen tension for phenylalanine biosynthesis. The maximum production occurred with an insufficient oxygen supply. More recent work by Choi and Tribe[6] has involved the systematic construction of multiple-mutation strains of *E. coli* in which key steps in metabolic regulation were altered (see Fig. 1). As the strain was prototrophic, it was not necessary to add relatively expensive nutrients to the growth medium.

The present research is concerned with the strain development of L-phenylalanine overproducer. Various regulatory mutants and auxotrophic regulatory mutants have been isolated from a wild type strain of *E. coli*. The fermentation results of these mutants have been compared in terms of L-phenylalanine accumulation and productivity.

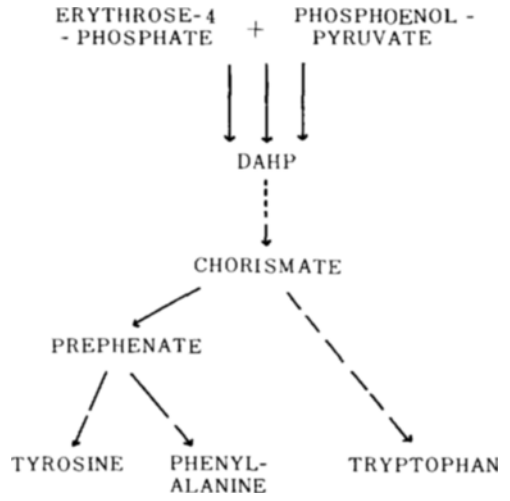

Fig. 1. Outline of biosynthesis of the aromatic amino acids in *E. coli*

MATERIALS AND METHODS

Microorganisms

The wild type strain of *E. coli* W3110 and its mutants derived in this laboratory were used. Stock cultures were maintained on nutrient agar slants at 4°C and subcultured weekly.

Chemicals

p-fluoro-DL-phenylalanine, ß-2-thienyl-DL-alanine, p-amino-DL-phenylalanine, 3-amino-L-tyrosine, and ampicillin were purchased from the Sigma. Ninhydrin was obtained from the Junsei Chemical Co. The other chemicals used were Reagent Grade.

Isolation of various mutants

A wide spectrum of mutation in *E. coli* can be induced by UV irradiation. The methods used for the isolation of various mutants were similar to that reported by Miller [7]. In Fig. 2 a survival curve is shown. As a survival of 0.1-1.0% is used for mutagenesis, 1 min of UV irradiation time was chosen for mutagenesis experiments.

In order to isolate regulatory mutants, *E. coli* W3110 grown on a Luria broth were centrifuged and resuspended in an equal volume of saline. After UV irradation for 1 min, 0.1 ml sample was taken in order to inoculate into 10 ml Luria broth, and allowed for expression of mutations. The cells were washed with saline and then spread out on glucose minimal plates containing phenylalanine or tyrosine analogue. Colonies arising after incubation of the plates for several days were picked up as regulatory mutants.

Tyrosine auxotrophic mutants were derived from phenylalanine analogue resistant mutants. Ampicillin at a final concentration of 20 μg/ml was treated two times for enrichment purposes [7].

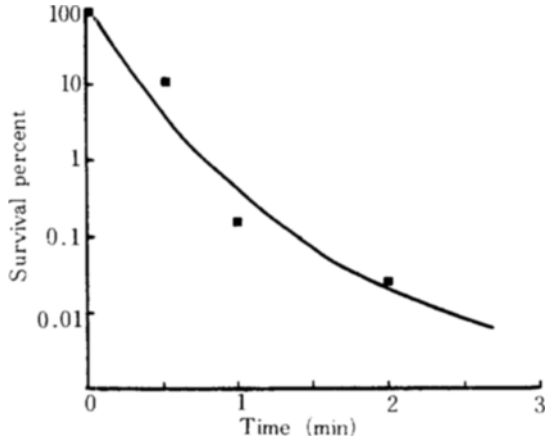

Fig. 2. Survival curve of *E. coli* W3110 with UV irradiation

Media and culture methods

The compositions of minimal and Luria media used for the isolation of various mutants, and fermentation medium[6,8,9] are shown in Table 1.

Table 1. Compositions of minimal, Luria and fermentation media

Component	Minimal	Luria	Jar Main	Jar Feed
Glucose	5 g/l	5 g/l	46.7 g/l	120 g
Sodium citrate	0.5 g/l		1.2 g/l	
$MgSO_4 \cdot 7H_2O$	0.1 g/l			
$(NH_4)_2SO_4$	1.0 g/l			
K_2HPO_4	7.0 g/l		0.75 g/l	1.125 g
KH_2PO_4	2.0 g/l			
Yeast Extract		10 g/l	0.75 g/l	
Tryptone		10 g/l		
NaCl		10 g/l		
Agar		(20 g/l)		
K_2SO_4			0.37 g/l	
$MgCl_2 \cdot 6H_2O$			0.80 g/l	
NH_4Cl			8.00 g/l	
$FeCl_3 \cdot 6H_2O$			0.015 g/l	
Stock Solution*			1 ml/l	

* Supplemented trace minerals; μM $(NH_4)_6Mo_7O_{24}$, 400 μM H_3BO_3, 10 μM $CuSO_4$, 80 μM $MnCl_2$, 10 μM $ZnSO_4$.

Unless otherwise specified, the cells were grown at 30°C for 8 h on an Erlenmeyer flask containing 100 ml of Luria. 100 ml of the inoculum was then transferred to a 1.5 l fermentation medium shown in Table 1. During the fermentation the feed medium of 0.4 l (300 g/l glucose plus 2.8 g/l K_2HPO_4) was added intermittently as a means of overcoming substrate inhibition effects, and the total additions of glucose was 95 g/l based on a final volume of 2 l.

A 5 l Jar fermentor (Marubishi, Japan) equipped with pH and temperature controllers was used. The pH was controlled at 7.0 by the automatic addition of 28% ammonia water and the temperature was controlled at 33°C. Agitation was provided by a flat-blade impeller (500-700 rpm) and aeration was 1 vvm. Dissolved oxygen tension was monitored and kept above 30% during the fermentation.

Analytical methods

L-phenylalanine concentration was estimated turbidimetrically after paper chromatographic separation [3,4]. Samples were appropriately diluted and spotted onto the Whatman No. 1 paper using 50 μl micropipettes. The developing reagent was prepared with n-butanol, acetic acid, and water (4:1:2). After ascending it up to 12 cm, 0.2% ninhydrin solution was

sprayed evenly for the detection of amino acids. The colored circle (R_f value of 0.57) was cutted and eluted using 5 ml of 50% ethanol, and then the absorbance was measured at 580 nm.

The methods for the measurements of cell growth and glucose were described in the previous papers [10-12].

RESULTS AND DISCUSSION

Effect of various analogues on the growth of *E. coli*

As suggested by several authors [4,13,14], ß-2-thienyl-DL-alanine (TA) and p-fluoro-DL-phenylalanine (PFP) are known as analogues of phenylalanine. In Fig. 3 the degree of growth inhibition for wild type strain by various phenylalanine or tyrosine analogues is shown. As shown in Fig. 3, both TA and PFP exert a great degree of inhibition on growth. However, 3-amino-L-tyrosine (3-AT) and p-amino-DL-phenylalanine (PAP, data not shown), tyrosine analogues, do not inhibit substantially. It is interesting to note that a mutant resistant to TA excreted large amounts of phenylalanine but a mutant resistant to PFP was observed to excrete only tyrosine [15]. For this reason TA was chosen as a phenylalanine analogue for the isolation of various phenylalanine resistant mutants.

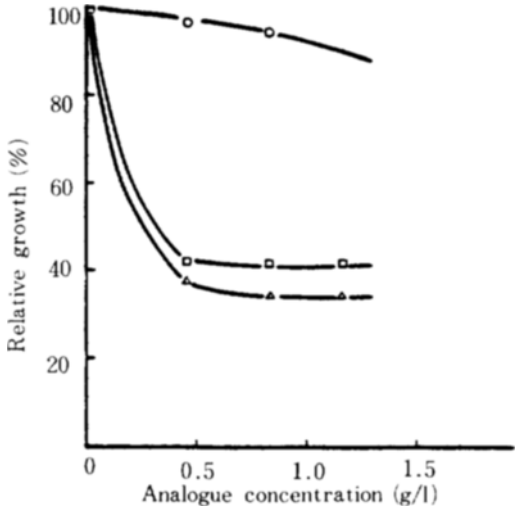

Fig. 3. Degree of growth inhibition for *E. coli* W 3110 by various phenylalanine or tyrosine analogues: (○) 3-AT, (□) TA, (△) PFP. 0.6 ml grown on minimal media was inoculated to 10 ml of minimal media containing analogue to be tested. Incubation was carried out for 9 h in test tubes at 30 °C, and then the absorbance was measured at 562 nm. The relative growth was determined from the absorbance against a control.

Regulatory mutants

In Table 2 various phenylalanine and/or tyrosine analogue resistant mutants obtained in this work are summarized together with their abilities for the production of L-phenylalaine. The strain TA6-7 which is resistant to TA (6 mg/ml) produced L-phenylalanine and a trace amount of L-tyrosine. The maximum L-phenylalanine concentration was 5.7 g/l in batch culture. Another strain TA10 which was isolated at a higher TA concentration of 10 mg/ml from the strain TA6-7 did not show any improvement of L-phenylalanine production.

When phenylalanine and tyrosine resistant mutants such as TA6-7AT1 and TA10PAP2 were used, the accumulation of L-phenylalanine was decreased and tyrosine was accumulated to some extent. It has been reported that the syntheses of the three 3-deoxy-*arabino*-heptulosonate 7-phosphate (DAHP) synthase (EC 4.1.2.15) isoenzymes are repressible by one or more of the aromatic amino acids [8,16,17]. Furthermore as the activities of the DAHP syntheses are each inhibitable by one of the aromatic amino acids [8,17], it appears that the decrease in L-phenylalanine accumulation by the mutants resistant to both phenylalanine and tyrosine analogues may be due to the formation of L-tyrosine which, in turn, inhbits one of the isoenzymes. Tribe and Pittard [8] reported that the activities of the isoenzymes decayed considerably in the stationary phase of growth,

Table 2. L-phenylalanine production by various regulatory mutants

Strains	Characteristics	Fermentation		
		Time (h)	Phenylalanine (g/l)	Productivity (g/l/h)
TA6-7	Phe^R	35	5.7	0.16
TA10	Phe^R	52	5.7	0.11
TA6-7 AT1	Phe^R Tyr^R	32	5.5	0.17
TA10 PAP2	Phe^R Tyr^R	47	4.4	0.09
W3110	Wild	30	0	0

while several other enzymes were found to be relatively stable.

Auxotrophic regulatory mutants

Based on our previous results and other findings [3,4,18,19], isolation of tyrosine auxotrophic regulatory mutants was attempted. Using a conventional replica plating method, three tyrosine auxotrophic mutants were isolated from the strain TA6-7 after mutagenesis with UV irradiation followed by two cycles of ampicillin treatment. These mutants were able to grow onto glucose minimal plates with L-tyrosine of 0.02 g/l but unable to grow onto glucose minimal plates. Among these three tyrosine auxotrophic mutants, however, only one (numbered TA6-7 Tyr134) had the property resistant to TA at a concentration of 6 mg/ml.

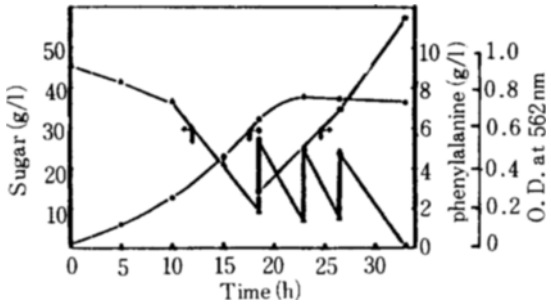

Fig. 4. Typical time course of L-phenylalanine fermentation in a 5 l Jar fermentor.

In Fig. 4 typical time course of L-phenylalanine fermentation in a 5 l Jar fermentor with *E. coli* TA6-7 Tyr134 is shown. As can be seen from Fig. 4, the fermentation was complete after 33 h cultivation and L-phenylalnine was accumulated up to 11.4 g/l. Therefore, both L-phenylalanine accumulation and productivity were increased significantly compared with the results obtained with strain TA6-7. Similar results were reported by several investigators [18,19], presumably due to the relief of DAHP synthase Tyr involved in metabolic pathway sown in Fig. 1.

Effects of tyrosine and tryptophan on L-phenylalanine production

The effect of tyrosine concentration on L-phenylalanine production was studied with the tyrosine auxotrophic regulatory mutant of *E. coli* TA6-7 Tyr134. As shown in Fig. 5, increasing the amount of tyrosine supplement resulted in decreased L-phenylalanine production. However, above the level of 25 mg/l tyrosine the L-phenylalanine accumulation was not affected by further supplement of L-tyrosine in the fermentation medium. As the maximum accumulation of L-phenylalanine was achieved without tyrosine supplement, it is believed that trace amounts of tyrosine

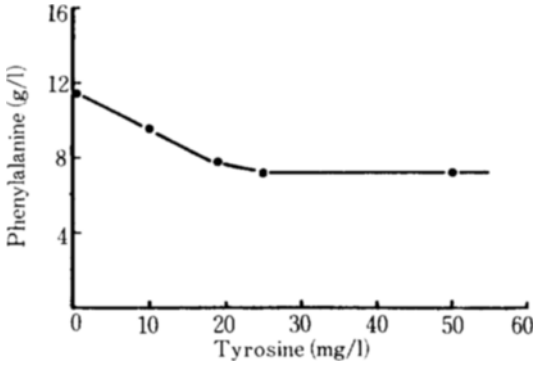

Fig. 5. Effect of tyrosine concentration on L-phenylalanine production.

for growth were sufficiently contained in other nutrients in seed and fermentation media. From the results shown in Fig. 5, it is likely that the DAHP synthase Tyr may still be repressed in excess tyrosine even though *E. coli* TA6-7 Tyr134 is used.

In Fig. 6 the effect of L-tryptophan concentration on L-phenylalanine fermentation is shown. Unlike the case of tyrosine effect, the L-phenylalanine accumulation was linearly decreased as the amount of L-tryptophan supplement was increased up to 80 mg/l. As suggested by Doy and Brown [17], the DAHP synthase Trp contributes 12% of the total DAHP synthases in *E. coli*. Therefore, it is highly probable that mutational blockage of the tyrosine and tryptophan pathways may enhance the yield of L-phenylalanine. This has been realized in our laboratory and the characteristics of double auxotrophic regulatory mutants will be the subject of a later publication.

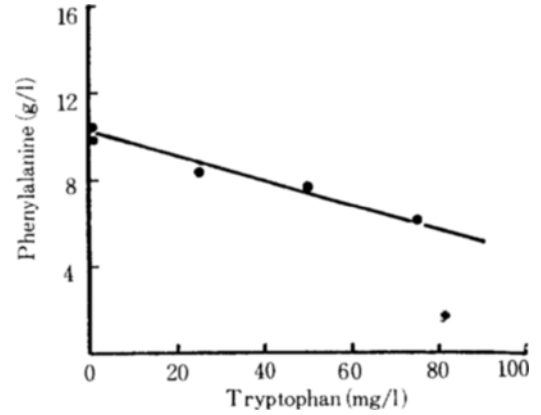

Fig. 6. Effect of tryptophan concetration on L-phenylalaine production. 25 mg/1 of tyrosine and 0.2 mg/1 thiamine were supplemented in the fermentation medium.

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