Kor. J. Appl. Microbiol. Bioeng.
Vol. 16, No. 5, 407-412 (1988)

Production of L-Tryptophan by Auxotrophs Derived from Analogue-resistant Mutants of *Escherichia coli*

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영양요구성 대장균 변이주를 이용한 L-트립토판 생산

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In order to increase the tryptophan productivity of *E. coli* SB1007, a mutant resistant to sulfanilamide was isolated and then a tyrosine auxotroph TY-90 was derived from the sulfanilamide-resistant mutant SA3-39-16. In the test-tube culture a quantitative amount of tryptophan was accumulated in strain TY-90 but in a jar fermentor culture the productivity was lower as compared to the level obtained by the parent strain. From the double auxotrophic mutant SB2756, a revertant resistant to 2,000μg/m*l* of β-thienylalanine, TA 40-10, was selected and then phenylalanine auxotrophs were derived from the revertant strain TA-40-10. One of the phenylalanine auxotrophs, TP-4, accumulated 3.7g/*l* of L-tryptophan after 71-hr cultivation in a jar fermentor experiment.

Recently, L-tryptophan has acquired increasing significance as a feed supplement for live-stock due to its very low content in most cereal grains. Although the current high price of L-tryptophan makes its addition to animal feeds difficult at this time, a reduction in tryptophan cost could result in increased use of this amino acid in the near future.

A number of different methods have been developed for the production of L-tryptophan which can be classified mainly into three processes; chemical, enzymatic, and microbial production methods. At the present time, L-tryptophan is produced by chemical or enzymatic process. Recently, however, much attention has been paid to the fermentative production of L-tryptophan by microbial processes(1). As a part of our efforts for microbial production of L-tryptophan, we have investigated the development of mutant strains and optimization of fermentation conditions using *Escherichia coli* (2).

Key words: L-Tryptophan, auxotrophic mutant, *E. coli*
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Initial studies on the tryptophan production by a recombinant cell, *E. coli* W3110 *tna* Δ *trp* EA/pCRT 185, had shown that the recombinant produced only small amount of tryptophan (30 mg/*l*) in spite of high copies of tryptophan operon genes. It was believed the tight regulatory functions in wild-type genes might be responsible for lower production of L-tryptophan. Thus we tried to isolate the analogue-resistant mutants using various analogues such as 5-methyltryptophan (5MT), 5-fluorotryptophan (5FT), β-2-thienylalanine (βTA), 3-aminophenylalaine (3AP), *p*-fluorophenylalanine (PFP) and 3-aminotryosine (3AT), as described in the previous report (2). The best producer, SB1007 strain, which was resistant to 5MT, 5FT and βTA, excreted 0.76 g/*l* of L-tryptophan in the test-tube culture. Much higher production of L-tryptophan by strain SB1007 than the recombinant cell implies that derepression of regulatory functions involved in tryptophan biosyn-

Table 1. List of strains.

Strains	Phenotype[a]	L-Tryptophan[b] (g/l)
SB1007	5MT^{r}5FT^rβTA^r	0.76
SA3-39-16	(SB1007) SA^r	0.91
SB2756	(SB1007) tyr$^-$ phe$^-$	2.60
TA-40-10	(SB2756)βTA^r tyr$^+$ phe$^+$	1.16
TY-90	(SA3-39-16) tyr$^-$	0.94
TP-4	(TA-40-10) phe$^-$	1.46

(a) 5MT; 5-methyltryptophan, 5FT; 5-fluorotryptophan, βTA; β-2-thienylalanine, SA; sulfanilamide
(b) L-Tryptophan production in the test tube culture.

thesis is prerequisite for overproduction of L-tryptophan in *E. coli*.

In this work, the isolation of auxotrophs from the analogue-resistant mutants and the production of L-tryptophan by newly isolated auxotrophic mutants have been investigated.

Materials and Methods

Bacterial strains

All bacterial strains used in this work are derived from *E. coli* W3110 and listed in Table 1.

Media

For the isolation of various mutants, minimal (3) and Luria (4) media were used. F medium contains per liter, glucose 50g, sodium citrate 1.2g, $MgCl_2 \cdot 6H_2O$ 1g, yeast extract 1g, NH_4Cl 10g, K_2HPO_4 3g, K_2SO_4 0.4g $FeCl_3 \cdot 6H_2O$ 15 mg, and trace elements solution 1 m*l*. Trace elements solution was composed of 30μM $(NH_4)_6Mo_7O_{24}$, 4 mM H_3BO_3, 0.1 mM $CuSO_4$, 0.8 mM $MnCl_2$, and 0.1 mM $ZnSO_4$. MFM medium denotes F medium in which yeast extract is not included. Glucose and the remaining components in the media were sterilized separately at 121 °C for 15 min. The initial pH of the media was adjusted to 7.0 with 2 N NaOH.

Isolation of mutants

Mutants were obtained after treatment with UV. The methods used for the isolation of various mutants are described elsewhere in detail (5).

Analytical methods

Glucose concentration was determined by DNS method (6). Indole and anthranilate were assayed using the method developed by Smith and Yanofsky(7). The DMAB method was used to analyze tryptophan(8). The concentration of tryptophan was also assayed by paper chromatograph. Optical density of the culture broth was determined at 540 nm with a spectrophotometer (Bausch & Lomb Spectronic 20). Dry cell weight was determined as described elsewhere (5).

Culture methods

For the test tube culture, a loopful of cells grown on agar slant was inoculated into test tubes containing 5 m*l* of F medium (with 2.5% $CaCO_3$). Bacteria precultured at 31 °C for 10 hrs in test tubes were inoculated (1 m*l*) into 100 m*l* of F medium (with 2.5% $CaCO_3$) in 500-m*l* shake flasks. Both test-tube and shake-flask cultures were carried out at 350 rpm and 31 °C in an incubator shaker (NBS Model G-25).

Jar fermentation was carried out in a 5-*l* jar fermentor (Shin-Young Engineering). The cells (100 m*l*) precultured at 31 °C for 10 hrs in shake flasks were transferred into fermentor containing 900 m*l* of fermentation medium. Air flow rate and agitation speed were 0.8-1.3 vvm and 600-800 rpm, respectively. The pH was controlled automatically at 7.0 with conc. NH_4OH. When glucose concentration in the medium was reduced to about 5 g/*l*, glucose (30 g) as well as KH_2PO_4 (1.5 g) was supplemented during the run. The dissolved oxygen concentration and the pH were monitored continuously.

Results and Discussion

Isolation of sulfanilamide-resistant mutants

According to the recent report by Shiio *et al* (9), sulfaguanidine-resistant mutants of both *Brevibacterium flavum* and *Corynebacterium glutamicum* showed about twofold higher tryptophan production than did the parent strains. Sulfonamides such as sulfaguanidine and sulfanilamide are structural analogues of p-aminobenzoic aicd (PABA), which is synthesized from chorismate. The rationale for use of sulfonamides as analogues in the breeding of tryptophan-hyperproducing strains is based on the fact that mutants resistant to sulfonamides could overproduce chorismate, an

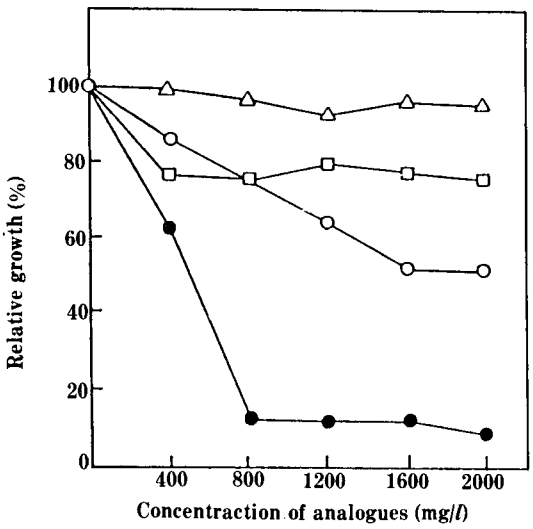

Fig. 1. Effects of various analogues on growth of strain SA3-39-16.
(△) 5MT, (□) 5FT, (○) SA, (●) βTA

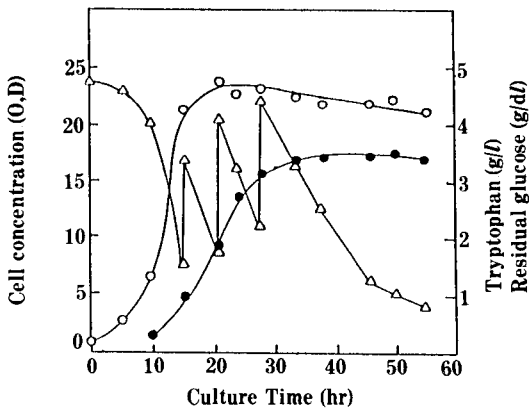

Fig. 2. Fed-batch fermentation of *E. coli* strain SA 3-39-16.
(○) cell, (△) residual glucose, (●) tryptophan

important intermediate in the pathway common for the biosynthesis of PABA and L-tryptophan. In this study, we have applied the same principle to *E. coli* in order to further increase the tryptophan productivity of the previously isolated mutant SB1007(2). Since the growth of strain SB1007 was almost completely inhibited by 100 μg/ml of sulfanilamide (SA) the mutants were isolated as colonies appearing on agar plates containing 500 to 1000μg/ml of sulfanilamide. One of sulfanilamide-resistant mutants, SA 3-39-16, produced 0.91 g/l of L-tryptophan in the test-tube culture.

Table 2. Effects of tyrosine and yeast extract on the production of L-tryptophan in shake-flask cultivation of TY-90 strain.

L-Tyrosine (mg/l)	Yeast extract (g/l)	Cell concentration (10dil.O.D.)	Tryptophan production (g/l)
0	0	<0.05	ND
	1	0.25	0.16
	2	0.41	0.32
50	0	<0.05	ND
	1	0.90	0.78
	2	0.53	0.34
100	0	1.10	0.74
	1	1.19	0.87
	2	0.62	0.41
200	0	1.15	0.62
	0.5	1.19	0.76
	1.0	1.35	1.22
	1.5	0.73	0.43
	2.0	0.60	0.41

ND; not determined

In Fig. 1, the degrees of growth inhibition for strain SA 3-39-16 by various analogues are shown. The results indicate that strain SA 3-39-16 has aquired enhanced resistance to sulfonamides while the analogue resistance (5MT, 5FT and βTA) of the parent strain SB1007 are retained.

A representive fermentation profile of strain SA3-39-16 in a jar fermentor is shown in Fig. 2. The tryptophan accumulation of strain SA 3-39-16 reached the mixmum amount, 3.5 g/l, after 35 hr and was not increased further. The amount of tryptophan formed in strain SA 3-39-16 was about the same that observed in the parent strain SB1007.

Tryptophan production by tyrosine auxotrophs derived from sulfanilamide-resistnat mutants

Using a conventional replica-plating method tyrosine auxotrophs were isolated from the sulfanilamide-resistant mutant SA 3-39-16 after mutagenesis with UV irradiation. The best producer, TY-90, accumulated 0.94 g/l of L-tryptophan in the test tube culture when F medium supplemented with 20 μg/ml of L-tyrosine was used. In the case of auxotrophs optimization of the required nutrient is necessary for efficient cell growth. Thus

the effect of L-tyrosine and yeast extract on the cell growth and tryptophan production was further examined in shake flasks.

As shown in Table 2, strain TY-90 was unable to grow in the MFM medium without addition of L-tyrosine. When yeast extract was supplemented in the MFM medium both the cell growth and tryptophan production were highest at 1.0 g/*l* of yeast extract independent of L-tyrosine concentration. The maximum tryptophan production, 1.22 g/*l*, was achieved in this experiment when the concentration of yeast extract and L-tyrosine were 1.0 g/*l* and 200 mg/*l*, respectively. A typical time course of cell growth and tryptophan production with a tyrosine auxotroph TY-90 are illustrated in Fig. 3. In this case the fermentation was carried at 35 °C, 1.15 vvm and 800 rpm in the F medium supplemented with 200 mg/*l* of L-tyrosine. As can be seen from Fig. 4, however, the amount of L-tryptophan produced by strain TY-90 (2.4g/*l*) was lower than the level produced by the parent strain SA3-39-16. Changes in the fermentation conditions and the nutrient compositions did not improve the tryptophan production in TY-90 strain.

Since tryptophan formation in strain TY-90 was not improved in a jar fermentor as compared to that in strain SB1007, we decided to use another strain SB2756 for the development of tryptophan-overproducing auxotrophic mutants. As mentioned earlier, strain SB2756 is a double auxotrophic mutant requiring both L-phenylalanine and L-tyrosine derived from strain SB1007. This auxotrophic mutant produced 3.5-fold higher amount of L-tryptophan than the parent strain SB1007 in the test-tube culture, but in a jar fermentor culture tryptophan production of SB2756 was lower than that of the parent (2), as was the case of TY-90. Therefore a revertant was first isolated from SB2756 and then auxotrophs were derived from it, as described in the followings.

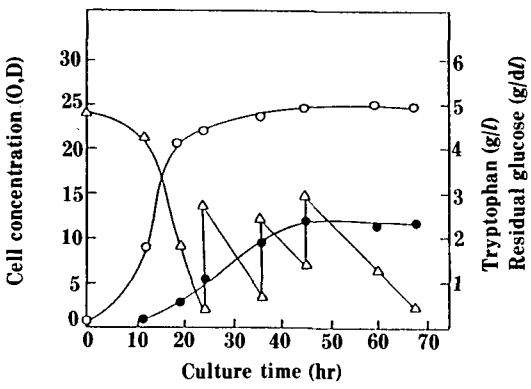

Fig. 3. Fed-batch fermentation of *E. coli* strain TY-90.
(○) cell, (△) residual glucose, (●) tryptophan

Isolation of a revertant from a double auxotrophic mutant

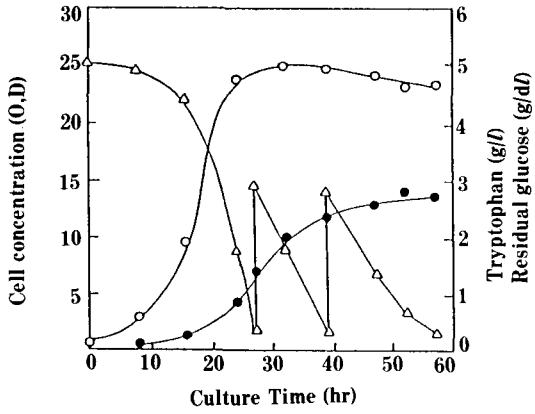

Fig. 4. Fed-batch fermentation of *E. coli* strain TA-40-10.
(○) cell, (△) residual glucose, (●) tryptophan

Table 3. *Effect of phenylalanine and tyrosine on cell growth and tryptophan production in shake-flask cultivation of TA-40-10 strain.*

Medium		Cell Growth (20 dil.O.D.)	Residual Glucose (g/*l*)	Tryptophan (g/*l*)	Final pH
Basal Medium	Concentration of Phe and Tyr (mg/*l* each)				
MFM	0	0.87	24.0	0.97	4.63
	10	0.85	25.8	0.97	4.66
	20	0.87	24.6	1.04	4.66
	50	0.87	25.2	1.08	4.67

Table 4. Effects of medium composition on tryptophan production in shake-flask cultivation of TP-4 strain.

Medium composition	Cell concentration (20 fold dil. O.D.)	Tryptophan production (g/l)	Product yield (%)
MFM	0.29	0.31	1.7
MFM + phenylalanine (20mg/l)	0.74	1.70	4.7
MFM + yeast extract (1.0g/l)	0.75	1.77	4.8
MFM + yeast extract (1.0g/l) + phenylalanine (20mg/l)	0.77	1.80	4.8
MFM + yeast extract (1.0g/l) + phenylalanine (40mg/l)	0.74	1.70	4.3

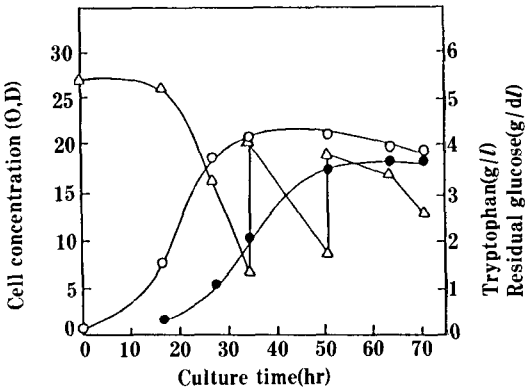

Fig. 5. Fed-batch fermentation of *E. coli* strain TP-4.
(○) cell, (△) residual glucose, (●) tryptophan

Revertant strains which do not require either L-phenylalanine or L-tyrosine for cell growth were isolated from a double auxotrophic mutant SB-2756. Because both SB1007 and SB2756 showed relatively low resistance to βTA (cf. Fig. 1) the strains resistant to 2000 mg/l of βTA were isolated among the revertant strains obtained by UV mutagenesis. The best tryptophan producer, named TA-40-10, accumulated 1.16g/l of L-tryptophan in the test-tube culture, about half of the amount produced by strain SB2756. When the effect of L-phenylalanine and L-tyrosine were examined in shake flasks, tryptophan production as well as cell growth were almost independent L-phenylalanine and L-tyrosine, as shwon in Table 3.

Fig. 4 shows the course of tryptophan production by strain TA-40-10 in a jar fermentor. The amount of tryptophan produced after 57-hr fermentation was about 2.7g/l. Further experimental results on the fermentation of TA-40-10 will be reported elsewhere (10).

Tryptophan production by phenylalaine auxotrophs

Using a similar method employed for the isolation of tyrosine auxotrophs several phenylalanine auxotrophs were derived from strain TA-40-10 and tested for L-tryptophan production. Among them strain TP-4, which was found to be a leaky auxotrophic mutant, produced L-tryptophan at the conentration of 1.46 g/l in the test-tube culture. As shown in Table 4, TP-4 was able to grow in the MFM medium in shake-flask culture although tryptophan production was only one-sixth of that in the medium supplemed with either L-phenylalanine or yeast extract.

When strain TP-4 was cultivated in a jar fermentor the amount of L-tryptophan produced after 71-hr fermentation was 3.7 g/l, as shown in Fig. 5. Contrary to the case of tyrosine auxotroph TY-90, the production of L-tryptophan by phenylalanine auxotroph TP-4 was 1.4-fold higher compared with the parent strain TA-40-10. However, cell growth and glucose consumption of TP-4 were lower than those of TA-40-10. Lower glucose consumption of auxotrophs as compared to that of the parent strain was common in all auxotrophic mutants including SB2756 and TY-90, which might reflect inefficiency of the metabolic activity of auxotrophs.

According to our experimental results it seems likely that higher tryptophan production by auxotrophic mutants, especially tyrosine auxotrophs, of *E. coli* are difficult as observed in the jar fermentation of TY-90 and SB2756. Further works on the optimization of fermentation conditions for auxotrophic mutants are now in progress.

요 약

트립토판 생산성을 향상시키기 위해 대장균 변이주인 SB 1007로부터 sulfanilamide 내성 균주를 유도하여 SA 3-39-16을 분리하고 이 균주로부터 tyrosine 영양요구주인 TY-90을 개발하였다. 시험관 배양시 TY-90균주는 모균주보다 트립토판 생산량이 우수하였으나 발효조 배양시에는 모균주에 비해 낮은 양의 트립토판이 축적되었다. 발효조에서의 생산성 증대를 위해 또 다른 대장균 변이주인 SB 2756으로부터 새로운 변이주의 개발을 모색하였다. 먼저 이중 영양요구성 균주인 SB 2756의 revertant를 분리하여 그중 2,000 mg/*l*농도의 β-thienylalanine에 내성을 갖는 TA-40-10을 분리하였고 이 균주로부터 phenylalanine 영향요구주인 TP-4를 개발하였다. 이 TP-4균주는 발효조에서 71시간 배양시 3.7g/*l*의 L-tryptophan을 생산하였다.

References

1. Shiio, I., in *Biotechnology of Amino Acid Production* (Ed. K. Aida, I. Chibata, K. Nakayama, K. Takinami, and H. Yamada), p. 188, Kodansha, Tokyo, 1986.
2. Lee, I.Y. and S.B. Lee, *Proc. lst Int'l Biotechnol. Symp.* p. 74, KABB, Seoul, Korea, 1985.
3. Davis, B.D. and E.S. Mingioli, *J. Bacteriol.*, **60,** 17 (1950).
4. Luria, S.E. and J.W. Burrows, *J. Bacterial.*, **74,** 461 (1957).
5. Lee, I.Y., M.S. Thesis, KAIST, 1985.
6. Miller, G.L., *Anal. Chem.*, **31,** 426 (1959).
7. Smith, O.H. and C. Yanofsky, *Methods in Enzymol.*, **5,** 794 (1962).
8. Smith, O.H. and C. Yanofsky, *J. Biol. Chem.*, **235,** 2051 (1960).
9. Shiio, I., S.-I. Sugimoto and K. Kawamura, *Agric. Biol. Chem.*, **48,** 2073 (1984).
10. Lee, I.Y., M.K. Kim, S.B. Lee, M.Y. Kwak H. Lee, submitted to *Kor. J. Appl. Microbiol. Bioeng.*

(Received September 12, 1988)