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Production of L-Threonine by Auxotrophs and Analogue Resistant Mutants of *Escherichia coli*

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영양요구성주 및 유사체 내성 대장균 변이주에 의한 L-스레오닌 생산

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Abstract — A threonine overproducer, *E. coli* TF427, which is resistant to threonine analogue, α-amino-β-hydroxyvaleric acid (AHV), and requires both methionine and isoleucine was developed by the mutations of *E. coli* W3110 using N-methyl-N'-nitro-N-nitrosoguanidine (NTG) and UV. The *E. coli* TF427 produced 46.5 g/*l* of threonine in a 5-L jar fermentor after 44 hr cultivation. The aspartokinase I of TF427 was not inhibited by threonine, and its synthesis was not repressed by threonine plus isoleucine.

L-Threonine is one of the most important essential amino acids and the addition of threonine into grains and feed increases the nutritional value of them. The biosynthetic pathway of threonine from aspartate and its regulatory mechanism have been well elucidated in *E. coli* K-12 (1-3). Aspartokinases and homoserine dehydrogenases are the key enzymes regulated by feedback inhibition and repression by threonine, isoleucine, methionine, or lysine. It has been reproted that auxotrophic mutants (4-7) which require methionine, diaminopimelic acid, or isoleucine and threonine analogue, α-amino-β-hydroxyvaleric acid (AHV), resistant mutants (8-10) produce L-threonine. In molecular genetic studies of threonine metabolism (11), the three structural genes coding for the threonine biosynthetic enzymes belong to a single *thr* operon, which consists of *thrA* (codes for aspartokinase I-homoserine dehydrogenase I complex enzyme; EC 2.7.2.4-EC 1.1.1.3), *thrB* (codes for homoserine kinase; EC 2.7.1.39), and *thrC* (codes for threonine synthase; EC 4.2.99.2). The expression of *thr* operon is regulated at the level of transcription termination via the attenuation mechanism, which is controlled by the intracellular concentration of threonine and isoleucine (12-14). Therefore, constitutive expression of *thr* operon is essential for the development of threonine overproducer suitable for industrial application.

In this paper, we describe the development of strain for L-threonine overproduction by the mutagenesis and discuss the mechanism of L-threonine overproduction by L-threonine producers.

Key words: Threonine, overproduction, aspartokinase I, regulatory mutant

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Materials and Methods

Bacterial strains, media, and culture conditions

The bacterial strains used in this study are shown in Table 1. LB medium was used for the routine growth and M9 medium was used as a mi-

Table 1. Threonine production by the mutants derived from *E. coli* W3110

Strain	Characteristics[a]	Threonine (g/*l*)[b]
W3110	Wild type	0.01
TF125	Met	0.4
TF219	Met , AHV^r (10 g/*l*)	5.7
TF313	Met , AHV^r (25 g/*l*)	15.3
TF427	Met , AHV^r (25 g/*l*), Ile^L	18.3

[a]Met , thionine auxotroph; AHV^r (10 g/*l*), resistant to 10 g/ of α-amino-β-hydroxyvaleric acid; AHV^r (25 g/*l*), re ant to 25 g/*l* of α-amino-β-hydroxyvaleric acid; Il isoleucine leaky character.
[b]The an nt of threonine was determined by HPLC.

nimal dium (15). If needed, M9 medium was supple nted with 100 μg/m*l* of L-methionine or 50 μg/r of L-isoleucine. Production medium was compo of 70g of glucose, 20g of $(NH_4)_2SO_4$, 1g of KH_2 , 0.5g of $MgSO_4 \cdot 7H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, 5 mg of $MnSO_4 \cdot 4H_2O$, 2g of yeast extract, 150 mg of L-methionine, and 30g of $CaCO_3$ in a total volume of 1 liter, pH 7.0 A loopful of cells grown at 33℃ on the LB agar plate was inoculated into 20 m*l* of production medium in 250 m*l* Erlenmeyer flask, and cultured with shaking at 33℃ for 48 hr. For the 5-L jar fermentor, fermentation medium was composed of 50g of glucose, 10g of $(NH_4)_2SO_4$, 2g of KH_2PO_4, 0.5g of $MgSO_4 \cdot 7H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, 5 mg of $MnSO_4 \cdot 4H_2O$, 2g of yeast extract, 150 mg of L-methionine in a total volume of 1 liter, pH 6.8. Seed culture was grown at 33℃ for 10 hr in 500 m*l* Erlenmeyer flask containing 75 m*l* of LB medium and then transferred to 1.5 *l* fermentation medium. During the cultivation the feed medium (glucose) was added intermittently. The culture conditions were following; pH was maintained at 6.8 with 28% ammonia water using an automatic pH controller, temperature at 33℃, aeration rate at 1 vvm, agitation speed at 750 rpm, and dissolved oxygen was maintained above 30%.

Isolation of auxotrophic mutants and AHV resistant mutants

E. coli mutants which require methionine or isoleucine were selected by the mutagenesis with UV irradiation and ampicillin enrichment (20 μg/m*l*) by using the procedures described by Miller (16) with some modifications. Culture of TF125 was treated with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) followed by growing at 33℃ for 2~4 days on minimal agar plates containing 2 mg/m*l* of AHV and 100 μg/m*l* of L-methionine. Colonies appeared on the plates were picked up as AHV resistant mutants. Mutants which are resistant to 15 mg/m*l* of AHV from TF219 were isolated on minimal agar plates containing 15 mg/m*l* of AHV and 100 μg/m*l* of L-methionine at 33℃ for 3~5 days.

Enzyme assay

Crude cell extract prepared from sonic disruption of cell grown in M9 minimal medium with appropriate supplements was used as an enzyme source. The aspartokinase I (AKI) activity was measured by the procedure of Truffa-Bachi and Cohen (17). One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of aspartyl-β-hydroxamate for 1 hr. The amount of protein was determined by the method of Bradford (18) with BSA as a standard.

Results and Discussion

Isolation of methionine auxotrophs and production of threonine

It has been reproted that methionine which is synthesized from homoserine, an intermediate of threonine biosynthesis, via cysthathionine (1) represses the synthesis of aspartokinase II (AKII)-homoserine dehydrogenase II (HDII) in *E. coli* (2). Thus, the blocking of homoserine flow to the methionine biosynthetic pathway and the lack of feedback repression of AKII-HDII by methionine might increase the production of threonine. Therefore, methionine auxotrophs were isolated from *E. coli* W3110 after mutagenesis with UV followed by ampicillin enrichment. Six methionine auxotrophs were selected and screened for their ability to produce threonine. All of them produced more threonine than *E. coli* W3110 (wild type). The secretion of threonine from the methionine auxotrophs seems to result from the derepression of AKII-HDII by limitation of methionine. Among these mutants, TF

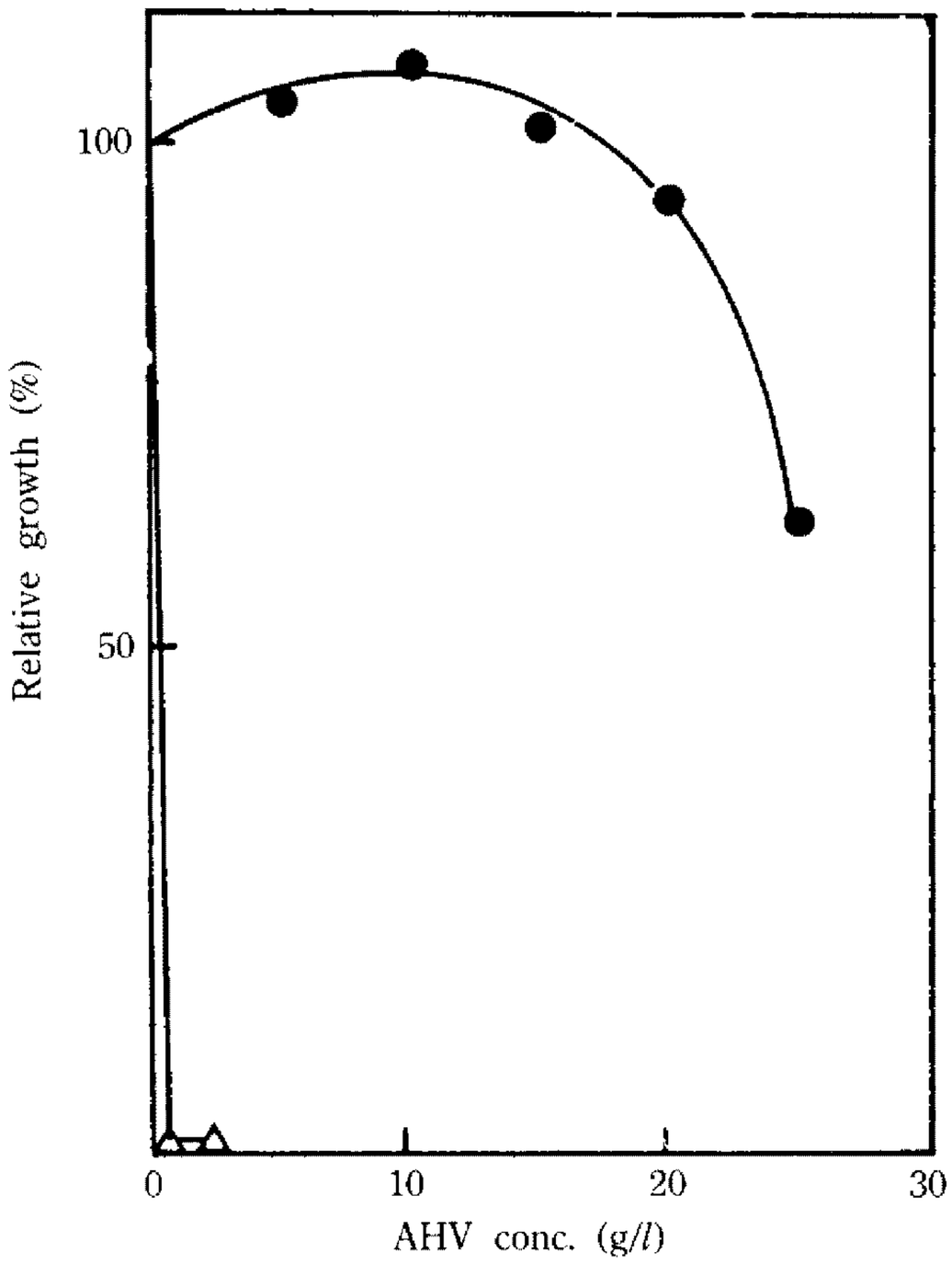

Fig. 1. Growth inhibition of *E. coli* TF125 and TF313 by AHV.
*Symbols; △−△, *E. coli* TF125; ●−●, *E. coli* TF313.

125 which produced 0.4 g/*l* of threonine (Table 1) was selected as the overproducer and used in the following experiments.

Isolation of AHV resistant mutants and production of threonine

The growth of TF125 was completely inhibited by the addition of AHV at the concentration more than 1 mg/m*l*. Thus, TF125 was mutagenized with NTG and selected on the agar plates containing 2 mg/m*l* of AHV. The frequency of appearance of AHV resistant mutants was about 1.6×10^{-5}. About 100 AHV resistant colonies were picked up and each colony was tested for the threonine productivity using production medium in 250 m*l* Erlenmeyer flask. 38 colonies of AHV resistant mutants produced more threonine than TF125. Among them, TF219 accumulated 5.7 g/*l* of threonine (Table 1). Although TF219 is resistant to 10 mg/m*l* of AHV, it is sensitive to 15 mg/m*l* of AHV. Therefore, the TF219 was mutagenized with NTG once again and selected on the agar plates containing 15 mg/m*l* of AHV. The frequency of appearance of high concentration of AHV resistant mutants was about 4.2×10^{-5}. About 100 mutants were tested for the threonine productivity by flask culture. Among them, TF313 which produced 15.3 g/*l* of threonine was selected as the best producer (Table 1).

The effect of AHV on the growth of TF125 and TF313 is shown in Fig. 1. TF313 showed a resistance up to 25 mg/m*l* of AHV, whereas TF125 was completely inhibited by the addition of 1 mg/m*l* of AHV.

AHV resistant strain, TF313, showed about 38-fold higher production of threonine than AHV sensitive strain, TF125. In order to elucidate the reason for overproduction of threonine by TF313, the AKI activity was measured. As shown in Table 2, AKI activity of TF313 was not inhibited by threonine and its expression was repressed by threonine plus isoleucine, whereas the AKI activity of TF125 was controlled by feedback inhibition and repression by threonine and threonine plus isoleucine, respectively. Therefore, it was clear that feedback resistant AKI is essential for the development of threonine producer.

Isolation of isoleucine auxotrophs and production of threonine

In *E. coli* the expression of *thr* operon is regulated by the intracellular concentration of isoleucine and threonine (12-14). Thus, the blocking of the biosynthetic pathway from threonine to isoleucine is essential for the overproduction of threonine. Based on this fact, isoleucine leaky and/or complete auxotrophs were selected by the mutagenesis with UV followed by ampicillin enrichment from TF313. Seven isoleucine leaky and/or complete auxotrophs were isolated and tested for the threonine productivity. Among them, TF427 produced 18.3 g/*l* of threonine (Table 1). In a 5-L jar fermentor (Fig. 2), the TF427 produced 46.5 g/*l* of threonine after 44 hr cultivation.

To confirm the TF427 is isoleucine auxotroph or not, the threonine deaminase, the first enzyme in the biosynthesis of isoleucine from threonine, activity was measured in TF313 and TF427. No de-

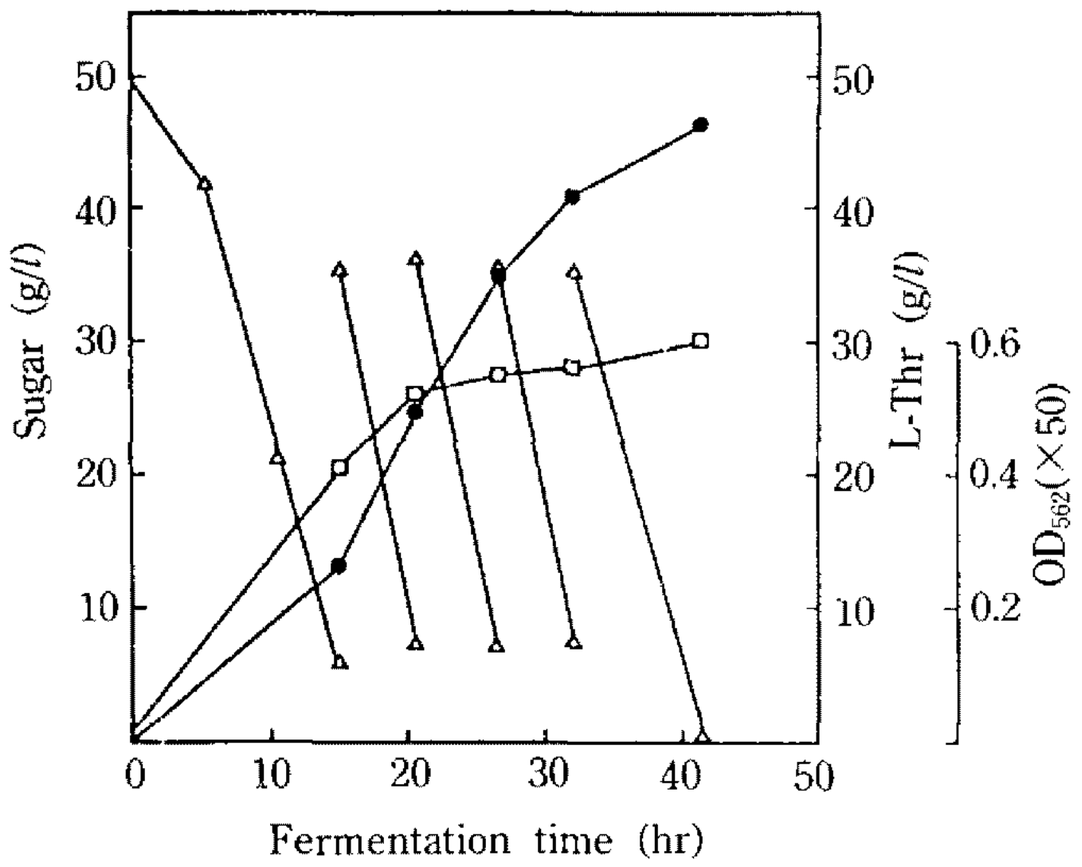

Fig. 2. Time course of threonine production of *E. coli* TF427 in a 5-L jar fermentor.
Culture conditions were described in the text.
*Symbols; △−△, sugar content; ●−●, threonine; □−□, OD_{562}(×50).

Table 2. Comparison of properties of aspartokinase I

Strain	Culture[a]	Specific activity (units/mg protein)	
		w/Lys[b]	w/Lys+Thr[c]
TF125	minimal	1.22	0.18
	excess	0.62	
TF313	minimal	2.37	2.23
	excess	1.19	
TF427	minimal	2.25	2.13
	excess	2.02	

[a] The minimal medium contained 20 mM lysine and methionine. The excess medium contained 20 mM lysine, methionine, threonine, and isoleucine.
[b] The activity was determined in the presence of 20 mM lysine.
[c] The activity was determined in the presence of 20 mM lysine and threonine.

tectable activity was observed in TF427. In contrast, TF313 showed normal threonine deaminase activity (data not shown). And TF427 grew slow on minimal agar plate containing only methionine. These results indicated that the TF427 is the isoleucine leaky auxotroph, which was caused by a mutation in the gene for threonine deaminase.

TF427 showed 20% higher threonine productivity than TF313 (Table 1). In order to elucidate the mechanism of threonine overproduction by TF427, the enzymatic properties of AKI were examined. As shown in Table 2, TF427 showed constitutive expression of AKI in high concentration of threonine plus isoleucine. The constitutive expression of AKI seems to result from *thr* attenuator mutation (13, 19), *ilvS* mutation (20), or *thrS* mutation (21). Furthermore, a mutation in *ilvA*, coding for threonine deaminase, leads to derepression of the *thr* operon, probably as a result of isoleucine limitation.

In order to develop the strain suitable for industrial production of threonine, we are now selecting another analogue resistant mutants or antibiotic resistant mutants.

요 약

대장균 W3110으로부터 NTG 및 UV를 사용하여 여러 단계의 돌연변이 실험을 거치면서 스레오닌 고생산균주인 대장균 TF427를 선별하였다. 선별된 변이주는 스레오닌 유사체인 AHV 내성, 메치오닌 및 이소루이신 요구성을 특징으로 한다. 5-L 발효조 실험에서 44시간 발효하였을 때 46.5 g/*l*의 스레오닌이 생산되었다. 효소분석에 의하면, TF427의 아스파토키나아제 I의 활성은 스레오닌에 의해 저해받지 않았으며, 이 효소의 합성은 스레오닌과 이소루이신에 의해 억제받지 않았다.

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