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Construction of L-Threonine Overproducing *Escherichia coli* by Cloning of the Threonine Operon

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The *thr* operon of *Escherichia coli* TF427, an α-amino-β-hydroxyvaleric acid (AHV)-resistant threonine overproducer, was cloned in a pBluescriptII KS$^+$ plasmid by complementation of *E. coli* mutants. All clones contained a common 8.8 kb *Hin*dIII-generated DNA fragment and complemented the *thrA*, *thrB*, and *thrC* mutants by showing that these clones contained the whole *thr* operon. This *thr* operon was subcloned in the plasmid vectors pBR322, pUC18, and pECCG 117, an *E. coli*/*Corynebacterium glutamicum* shuttle vector, to form recombinant plasmids pBTF 11, pUTF25 and pGTF18, respectively. The subcloned *thr* operon was shown to be present in a 6.0 kb insert. A transformant of *E. coli* TF125 with pBTF11 showed an 8~11 fold higher aspartokinase I activity, and 15~20 fold higher L-threonine production than TF125, an AHV-sensitive methionine auxotroph. Also, it was found that the aspartokinase I activity of *E. coli* TF125 harboring pBTF11 was not inhibited by threonine and its synthesis was not repressed by threonine plus isoleucine.

Gene cloning is an important tool in biotechnology and has proven to be a useful technique for strain development of various amino acids overproduction, such as phenylalanine (21), tryptophan (1), lysine (19) and threonine (6).

In *E. coli* K-12, the biosynthesis of threonine requires the expression of the *thr* operon, which consists of *thrA* (codes for aspartokinase I-homoserine dehydrogenase I complex; 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 the *thr* operon is regulated at the level of transcription termination by an attenuation mechanism which is controlled by the intracellular concentration of threonine plus isoleucine (9, 23). Regulatory mutants affecting the expression of the *thr* operon have mutations in the *thrO* region, located proximal to the *thrA* structural gene (4, 20). Molecular cloning of the *thr* operon to improve threonine production by expression of the *thr* operon in *E. coli* has been reported (15, 16), although the problem of stable plasmid maintenance still remains to be overcome (16, 17). Previous work on the construction of threonine-overproducing strains of *E. coli* enabled us to isolate the AHV-resistant threonine overproducer, *E. coli* TF427 (11). The aspartokinase I activity of TF427 was not inhibited by threonine. Also, it was found that the expression of aspartokinase I (*thrA1* gene), which is the first structural enzyme of the threonine operon, was not repressed by threonine plus isoleucine.

In this study, we investigated the cloning of the *thr* operon from AHV-resistant threonine overproducer *E. coli* TF427, and its expression in threonine producers.

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Key words: *Escherichia coli*, threonine operon, cloning, expression, fermentation

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are listed in Table 1.

Growth Conditions and Fermentation of L-threonine

Strains were routinely grown in LB or M9 minimal media (13) with appropriate supplements. The following supplements were added to the minimal medium, if necessary, at a final concentration of 50 μg/ml of amino

Table 1. Bacterial strains and plasmids used in this study.

Strain/Plasmid	Characteristics	Source
E. coli		
TF125	Met^-	This Lab.
TF427	Met^-, AHV^r, Ile^L	This Lab.
Gif 106M1	*thrA, rpsL9, malT1, metLM*1000 *xyl-7, mtl-2, ilvA*296, *arg*1000, *lysC*1001, *thi*-1	Theze & Saint-Girons (23)
Hfr 3000 YA73	*thrB, relA1, spoT1, thi*-1	Theze & Saint-Girons (23)
Gif 41	*thrC, relA1, spoT1, thi*-1	Theze & Saint-Girons (23)
Plasmids		
pBluescriptII KS^+	Ap^r, 2.9 kb	STRATAGENE*
pTF23 & pTF32	Ap^r, 11.7 kb, pBluescriptII KS^+ carrying 8.8 kb insert containing *thr* operon	This work
pTF231	Ap^r, 8.9 kb, pBluescriptII KS^+ carrying 6 kb insert containing *thr* operon	This work
pTF2311	Ap^r, 7 kb, *thrC* deleted pTF231	This work
pBTF11	Ap^r, 10 kb, pBR322 carrying *thr* operon	This work
pUTF25	Ap^r, 8.7 kb, pUC18 carrying *thr* operon	This work
pUTF251	Ap^r, 6.7 kb, *thrC* deleted pUTF25	This work
pECCG117	Km^r, 5.8 kb, *E. coli/C. glutamicum* shuttle vector	This Lab.
pGTF18	Km^r, 11.8 kb, pECCG117 carrying *thr* operon	This work

Abbreviations: Met; methionine, Ile; isoleucine, Ap; Ampicillin, Km; Kanamycin, AHV; α-amino-β-hydroxyvaleric acid.
*STRATAGENE cloning vector

acids and 1μg/ml of thiamine-HCl. Ampicillin(Ap) or kanamycin(Km) was used at a final concentration of 50 μg/ml. Cells used for the production of threonine were grown in a flask containing 20 ml of fermentation medium (70 g of glucose, 20 g of $(NH_4)_2SO_4$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, 5 mg of $MnSO_4 \cdot 4H_2O$, 2 g of yeast extract, 150 mg of methionine, and 30 g of $CaCO_3$ in a total volume of 1 liter, pH 7.0). A loop of cells grown at 33℃ on LB agar plates, or LB agar plates with antibiotics, was inoculated into 20 ml of fermentation medium and cultured with shaking at 33℃ for 50 h. The amount of threonine was measured using HPLC (Waters Associates, USA). The column temperature of the Waters amino acid analyzer and flow rate were 61℃ and 0.5 ml/min, respectively.

Isolation and Manipulation of DNA

Plasmids were isolated from *E. coli* strains by the method of Birnboim and Doly (2). Chromosomal DNA was isolated from *E. coli* by the procedure of Marmur *et al.* (14). Restriction endonuclease digestion and ligation with T4 DNA ligase were done using standard procedures (13).

Construction of an *E. coli* Gene Library

Total chromosomal DNA from *E. coli* TF427 was partially digested with *Hin*dIII, and DNA fragments ranging from 8 to 12 kb were isolated from LMP agarose gel. 3 μg of *Hin*dIII-digested chromosomal DNA fragments were ligated with *Hin*dIII-digested, dephosphorylated pBluescriptII KS^+ at 15℃ for 15 h. This ligation mixture was used to transform *E. coli* Hfr 3000 YA73.

Transformation

Transformation of *E. coli* cells was carried out either by the $CaCl_2$ method (5) or by electroporation (7).

Preparation of Crude Extract and Assay of Aspartokinase I

Cells were grown to the exponential phase at 33℃ in M9 minimal medium. Cell pellets harvested from 100 ml of culture medium were washed with buffer A (0.02 M potassium phosphate , pH 7.2, containing 0.15 M KCl) and suspended in 1 ml of buffer A, then sonicated for 30 sec at 4℃. Cell debris was removed by centrifugation at 36,000×g for 30 min and the supernatant was used as an enzyme source. Aspartokinase I activity in the crude extracts was assayed according to the method of Truffa-Bachi and Cohen (24). One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of aspartate-β-hydroxamate in 1 h. Protein concentration was determined by the Bradford method (3).

Stability of Plasmids

The stability of plasmids was examined after cultivation in the fermentation medium. The stability was determined by transferring cells grown on LB agar to LB agar containing antibiotics and then counting the number of

colonies which grew on the LB agar plates containing antibiotics.

RESULTS AND DISCUSSION

Cloning of *thr* Operon

Chromosomal DNA was isolated from *E. coli* TF427, which is an AHV-resistant threonine overproducer. The chromosomal DNA was partially digested with *Hin*dIII and ligated into the unique *Hin*dIII site of pBluescriptII KS$^+$. The ligated mixture was used to transform *thrB* auxotrophic *E. coli* Hfr 3000 YA73. Thr$^+$ transformants were selected by growth on M9 minimal medium lacking threonine. Plasmid DNA was isolated from these transformants and subjected to restriction enzyme analysis, followed by agarose gel electrophoresis. It was found that *thrB* complementing plasmids contained an 8.8 kb *Hin*dIII fragment spanning the *thrB* gene region of the *E. coli* chromosome. The presence of the *thrA* and *thrC* genes was confirmed by complementation of *thrA* and *thrC E. coli* mutants Gif 106M1 and Gif 41. The plasmids complementing all *thrA, thrB* and *thrC E. coli* mutants were designated as pTF23 and pTF32. Plasmid pTF32 was different from plasmid pTF23 only in the orientation of the *thr* operon in pBluescriptII KS$^+$. An essential region of *thr* operon was obtained as about 6.0 kb which was confirmed by complementation tests with *thrA, thrB* and *thrC E. coli* mutants (Fig. 2). The *thr* operon, obtained as a 6 kb *Hin*dIII-*Bam*HI fragment from plasmid pTF23, was joined with *Hin*dIII-*Bam*HI-digested plasmid pBR322 and *Hin*dIII-*Bam*HI-digested plasmid pUC18 to form plasmids pBTF11 (Fig. 1) and pUTF25. From plasmid pTF23, plasmid pTF231 was obtained by subcloning and reducing the fragment size. Plasmid pTF2311 was obtained by deleting the *thrC* gene from plasmid pTF231 by *Eco*RI digestion followed by self ligation. Plasmid pGTF18 was constructed by joining the 6 kb *Bam*HI-*Kpn*I fragment isolated from plasmid pTF231 to *Bam*HI-*Kpn*I-digested plasmid pECCG117 (18) (Fig. 2).

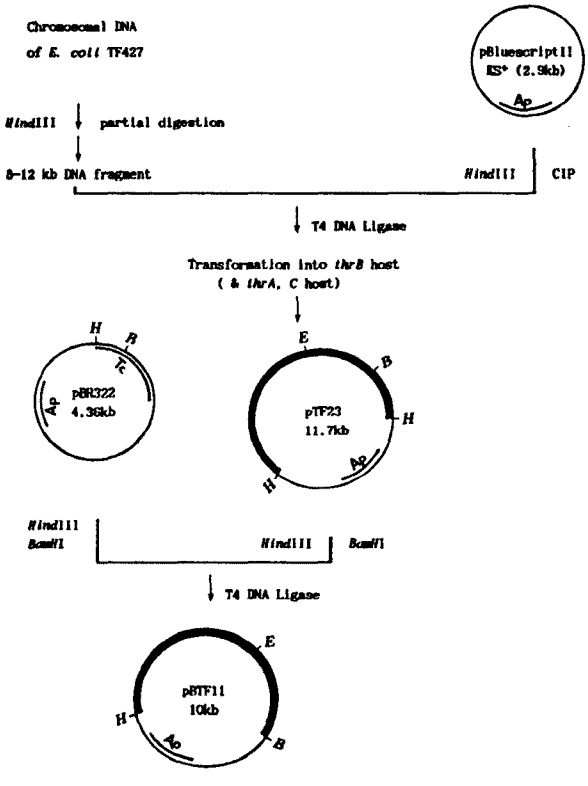

Fig. 1. Schematic diagram for the construction of plasmids pTF23 and pBTF11.

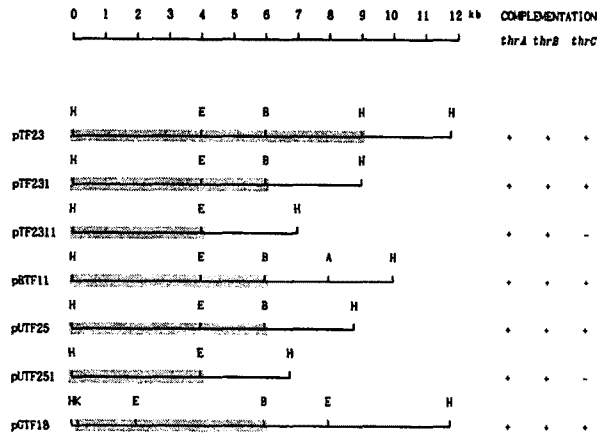

Fig. 2. Restriction map of plasmid pTF23 and subcloning of the threonine operon on pBR322, pUC18 and pECCG117.

— indicates the *E. coli* TF427 chromosomal DNA fragment containing the threonine operon. Abbreviations: H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; A, *Ava*I; K, *Kpn*I.

Expression of the Threonine Operon in *E. coli*: Repression and Inhibition of Aspartokinase I by Amino Acids

The *thr* operon of *E. coli* K-12 is composed of four structural genes (*thrA1A2BC*) (23) and a regulatory region (*thrO* region) which is located before *thrA1* (10). The regulatory region, which has been called the *thr* attenuator appears approximately 30 base pairs upstream the *thrA1* gene. All the regulatory mutations that have been identified by DNA sequencing lie within the attenuator (12). The mutations influence the efficiency of transcription termination at the attenuator region *in vitro* and *in vivo* (22). In *E. coli* K-12, the expression of the *thr* operon is regulated by the intracellular conce-

ntration of both threonine and isoleucine, through the attenuation mechanism. The two enzymes of aspartokinase I-homoserine dehydrogenase I encoded by the *thrA 1* and *thrA2* genes, are carried by a single polypeptide chain, and inhibited by threonine (8). It has been shown that the donor strain of the mutated *thr* operon, *E. coli* TF427 constitutively expressed aspartokinase I and the activity of aspartokinase I was not inhibited by threonine (11). Therefore, provided that the other enzymes encoded by this *thr* operon would have the same level of enzyme expression, the gene dosage effect could contribute to the improvement of threonine production in *E. coli*. To verify the gene dosage effect of the *thr* operon, the specific activity of one of the gene products, aspartokinase I, was examined using *E. coli* mutant and recombinant strain. As shown in Table 2, the aspartokinase I activity of recombinant strain *E. coli* TF125/pBTF11, was 8-11 fold higher than the aspartokinase I activity of TF125. This indicates that the *thr* operon is well expressed in TF125, regardless of the regulation of threonine and isoleucine. The level of aspartokinase I activity in TF125/pBTF11 increased with the gene dosage effect. Because the threonine operon was cloned in plasmid pBR322 which is present in about 15~20 copies per chromosome under normal conditions (13). A complete understanding of constitutive expression of this mutated *thr* operon must include the knowledge of the nucleotide sequence of the regulatory region of the *thr* operon.

Production of L-threonine in *E. coli* Strains

The *thrB* host was transformed with pTF23, pTF231, pTF2311, pBTF11, pUTF25 and pGTF18, and fermentations were carried out using these strains in the presence of antibiotics. All of the transformants produced about 0.2 to 0.5 g/*l* of L-threonine in a fermentation medium (data not shown), although the host itself did not produce L-threonine and did not grow in minimal medium without threonine. Because the L-threonine produced by the recombinant strain could also be used for the growth of the *thrB* host itself, L-threonine did not much accumulate in the fermentation broth.

In order to increase the production of L-threonine, *E. coli* TF125 was transformed with pTF231, pUTF25, pGTF18 and pBTF11 and fermentations were carried out using these strains. As shown in Table 3, the threonine production of TF125 harboring pTF231 or pUTF25 was not much greater than that of TF125. However, TF125/pBTF11 showed a resistance to 25 mg/m*l* of AHV and produced 12.5 g/*l* of L-threonine after 50hr cultivation in a flask containing 20 m*l* of fermentation medium, whereas the AHV-sensitive host strain, TF125, produced only 0.66 g/*l* of L-threonine. It is clear that the greater L-threonine production of TF125/pBTF11 is due to the gene dosage effect of the threonine biosynthetic enzymes encoded by the threonine operon, and the stable maintenance of the recombinant plasmid during cultivation. Plasmids pTF231 and pUTF25 were unstable in TF125 and, as a result, more than 99.9% of all cells lost the plasmids after 50 hr cultivation in the fermentation medium. In the case of TF125/pBTF 11, more than 90% of the cells were found to be Ap-resistant in the selective fermentation medium. Because ampicillin added to the fermentation medium as a selective pressure could not act throughout the course of the fermentation by degradation with extracellular β-lactamase that had leaked out from the cells (16), plasmid pGTF18 with Km as a marker was used to transform TF125. However, TF125 transformed with pGTF18 did not produce more threonine than TF125 in spite of the stable maintenance of the plasmid throughout the course of the fermentation.

In order to further increase L-threonine production, pBTF11 was used to retransform *E. coli* TF427. However, TF427/pBTF11 produced the same amount of L-threonine as TF427. After fermentation for 50 hr under

Table 2. Comparison of the aspartokinase I activity.

Strain	Growth medium	Specific activity of aspartokinase I (μ/mg protein)	
		Case 1	Case 2
E. coli TF125	ML*	1.02	0.18
	TIML**	0.60	
E. coli	ML	7.05	6.52
TF125/pBTF11	TIML	6.03	

*Cells were grown in M9 medium containing 20 mM each methionine (M) and lysine (L).
** Cells were grown in M9 medium containing 20 mM each methionine, lysine, threonine (T) and isoleucine (I).
Case 1: Specific activity of aspartokinase I was determined in the presence of 20 mM of lysine.
Case 2: Specific activity of aspartokinase I was determined in the presence of 20 mM each lysine and threonine.

Table 3. Production of L-threonine by various recombinant *E. coli* strains.

Strain	Plasmid	Threonine (g/*l*)	Plasmid stability(%)
TF125	none	0.66	–
TF125	pTF231	0.74	≪0.01
TF125	pUTF25	0.46	≪0.01
TF125	pGTF18	0.65	>90
TF125	pBTF11	12.5	>90
TF427	none	18.5	–
TF427	pBTF11	18.3	≪0.01

selective conditions (50 μg/ml of Ap in the fermentation medium), more than 99% of all cells lost the plasmids. These results suggest that the stable maintenance of the recombinant plasmid is important to improve the production of threonine in the hyperproducing host cell.

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