Construction of L-Threonine Overproducing Strains of *Escherichia coli* K-12 Using Recombinant DNA Techniques

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The threonine operon of *Escherichia coli* was cloned in plasmid pBR322, using a threonine producing mutant, β IM4, as the DNA donor. A recombinant plasmid, pAJ294, that contains the whole threonine operon was obtained. No. 29-4, a transformant of β IM4 with pAJ294, had about eleven copies of pAJ294, five times higher homoserine dehydrogenase activity (coded by the *thrA* gene), and about three times higher threonine productivity than those of β IM4. No. 29-4 produced 13.4 g/liter of L-threonine from 30 g/liter of glucose in the culture medium.

Threonine biosynthesis is known to be regulated by two mechanisms in *E. coli*. One is feedback inhibition by L-threonine of a bi-functional enzyme, aspartokinase I (AK-I)-homoserine dehydrogenase I (HDH-I), and another is multivalent repression of the threonine biosynthetic enzymes by L-threonine plus L-isoleucine¹⁾ through the attenuation mechanism.^{2,3)} We have previously constructed threonine producing mutants of *E. coli*.⁴⁾ These mutants have an altered AK-I-HDH-I which is less sensitive to the feedback inhibition by L-threonine, and also have an isoleucine auxotrophic mutation that may cause derepression of threonine genes under isoleucine-limiting conditions.

In order to increase the productivity of threonine by amplifying the threonine genes, we employed recombinant DNA techniques. In this paper we will describe the construction of improved L-threonine producing strains and some of their properties.

MATERIALS AND METHODS

Bacterial strains and plasmid. The bacterial strains used were derived from *E. coli* K-12. Threonine overproducing

mutant β IM4⁴⁾ was used as the DNA donor and the recipient. Threonine auxotrophic mutants, No. 1 *thrA*, No. 43 *thrB*, No. 313 *thrC*, No. 44 *thr* (not identified) and No. 255 *thr* (not identified), were derived from β IM4 and used as the recipients. Plasmid pBR322⁵⁾ was used as the vector.

Media. L medium⁶⁾ and Davis⁷⁾ minimal medium were used. Amino acids (100 μ g/ml), thiamine-HCl (1 μ g/ml), ampicillin (100 μ g/ml) and tetracycline (20 μ g/ml) were added when required. ET medium containing 3% glucose, 1% (NH₄)₂SO₄, 0.2% KH₂PO₄, 0.1% MgSO₄·7H₂O, 2 ppm Fe²⁺, 2 ppm Mn²⁺, 1 μ g/ml thiamine-HCl, 300 μ g/ml L-proline, 100 μ g/ml L-isoleucine, 100 μ g/ml L-methionine and 2% CaCO₃ (sterilized separately) was used for L-threonine production.

Preparation of DNA. Chromosomal DNA was prepared from strain β IM4 using the method of Saito and Miura⁸⁾ with modifications as follows. Cells were cultured in 1 liter of L medium at 37°C for 3 hr to the exponential growth phase and then harvested by centrifugation. The packed cells were resuspended in 25 ml of buffer containing 0.1 M EDTA and 0.1 M Tris-HCl (pH 9.0). SDS was added to a final concentration of 1%, and the lysate was incubated at 60°C for 10 min. After phenol extraction of the viscous lysate, the DNA was precipitated by adding 2 volumes of ethanol and spooled out on a glass rod. The DNA was further purified by digestion with preheated pancreatic RNase and self-digested pronase, phenol extraction and ethanol precipitation, and then dissolved in TEN buffer; 20 mM Tris-HCl (pH 8.0)/20 mM NaCl/1 mM EDTA.

Plasmid DNA was prepared in essentially the same way as described by Tanaka and Weisblum.⁹⁾

Enzymes. Restriction enzyme digestion and ligation of DNA were done as suggested by the suppliers. The following commercial preparations of enzymes were used: *EcoRI*, *HindIII*, *PstI*, *BamHI*, T4 DNA ligase (Boehringer) and *SalI* (Miles).

Homoserine dehydrogenase activity was assayed by the method of Truffa-Bachi *et al.*¹⁰⁾

Transformation. Recipient strains were transformed using the method of Lederberg *et al.*¹¹⁾

Determination of plasmid copy number. The copy number of the plasmid was determined by the CsCl-EtBr density gradient centrifugation method. Cells were cultured in 3 ml of minimal medium supplemented with casamino acid (0.5%), thiamine-HCl (1 µg/ml), [³H]-thymidine (10 µCi; 1.6 µM) and deoxyadenosine (200 µM) at 30°C for 16 hr, harvested by centrifugation, and washed twice with TEN buffer. The packed cells were resuspended in 0.5 ml of TEN buffer containing 2 mg/ml of lysozyme and 50 mM EDTA. After this suspension was incubated at 37°C for 50 min, preheated pancreatic RNase (to a final concentration of 50 µg/ml) was added and incubated for a further 30 min. Then self-digested pronase (1 mg/ml) and Sarkosyl (0.6%) were added and the mixture was incubated at 37°C for 2 hr. Using a small portion of this lysate, the total radioactivity (5% ice-cold TCA precipitable) was determined. Then covalently closed circular plasmid fraction was separated by CsCl-EtBr gradient centrifugation in a Hitachi PR65T angle rotor at 38,000 rpm for 40 hr, and its radioactivity was measured.

Cultivations for L-threonine production. A loopful of cells grown at 30°C for 24 hr on L agar (supplemented with ampicillin or tetracycline when necessary) was inoculated into 20 ml of ET medium in a 500 ml flask, and cultured at 30°C for 3 days with shaking. The amount of L-threonine in the culture broth was determined by a microbioassay with *Leuconostoc mesenteroides* ATCC 8042 and with an amino acid analyser.

Agarose gel electrophoresis. Electrophoresis was carried out using 0.8% agarose gel with a Tris-acetate buffer system as described by Sharp *et al.*¹²⁾

Halo test. Excretion of L-threonine from colonies was simply determined by the halo formation test. Strain No. 43, a threonine auxotrophic mutant, was used as the indicator. It was spread on minimal medium agar plates, and strains to be tested were spotted on the plate. After incubation at 30°C for 1~2 days the satellite growth of the indicator strain around the colonies was observed.

RESULTS

Cloning of threonine genes

Twenty-one µg of βIM4 chromosomal DNA and 12 µg of pBR322 DNA were digested with *HindIII*, mixed and ligated as described in MATERIALS AND METHODS, then the ligation mixture was used to transform threonine auxotrophic strains, Nos. 43, 44 and 255. Presumable transformants that grew on minimal agar supplemented with L-proline, L-isoleucine, L-methionine and thiamine-HCl were picked up and tested for ampicillin resistance, L-threonine excretion and the existence of plasmids larger than the vector pBR322. Twenty-two transformants were obtained. All the plasmids obtained from them were almost the same size, about 13 kb.

To confirm the clone, plasmid DNA was extracted from one of the 22 transformants and used for re-transformation of a *thrB* mutant, No.43. The transformants were selected by Amp^r, and five of them were further examined for their Thr⁻ phenotype. All of the five were found to be Thr⁺, thus the plasmid was concluded to be a pBR322-*thr* recombinant plasmid.

Production of threonine by Thr⁺ transformants

Threonine productivities of the Thr⁺ transformants were examined by cultivation in ET medium (Fig. 1). Among 22

FIG. 1. Distribution of Threonine Productivities among the Thr⁺ Transformants.

Twenty-two Thr⁺ transformants were cultured in ET medium at 30°C for 70 hr. The arrow in the figure shows the amount of L-threonine produced by strain βIM4.

transformants, strain No. 29 had the highest productivity; 5.4 g/liter of L-threonine, while β IM4 produced 2.9 g/liter.

Transformation of β IM4 with the pBR322-*thr* recombinant plasmid

In order to improve the productivity of threonine, the pBR322-*thr* recombinant plasmid was introduced into the parental threonine producing strain, β IM4. The recombinant plasmid DNA was prepared from transformant No. 29 described above, and was used to transform β IM4 to Amp^r. About 70% of the transformants showed higher L-threonine productivities than β IM4 as checked by the halo formation test. From those high producers, 14 transformants were picked up and cultured in ET medium (Fig. 2). The best

FIG. 2. Distribution of Threonine Productivities among the Transformants of β IM4, Harboring the pBR322-*thr* Plasmid.

The 14 transformants (see text) were cultured in ET medium at 30°C for 72 hr. The arrows (1) and (2) in the figure show the amounts of L-threonine produced by β IM4 and strain No. 29, respectively.

result was 9.0 g/liter of L-threonine accumulated by No. 29-4.

Threonine production by recombinant strain No. 29-4

By checking the effects of components of ET medium on L-threonine production of No. 29-4, the following ET-II medium was devised; 3% glucose, 1% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.1% MgSO₄·7H₂O, 2 ppm Fe²⁺, 2 ppm Mn²⁺, 1 mg/ml L-aspartate, 450 μ g/ml L-proline, 100 μ g/ml L-isoleucine, 100 μ g/ml L-methionine, 1 μ g/ml thiamine-HCl, 2 mg/ml yeast RNA and 4% CaCO₃ (pH 7.0).

Figure 3 shows the time course of L-threonine production by strain No. 29-4. After cultivation for 72 hr, 13.4 g/liter of threonine was accumulated in the culture broth. Amino acids in the broth were assayed using an amino acid analyser (Table I). By-production of

FIG. 3. Time Course of L-Threonine Production by Recombinant Strain No. 29-4.

Strain No. 29-4 was cultured in 20 ml of ET-II medium in flasks. —●—, L-threonine accumulated, —○—, residual glucose.

TABLE I. ACCUMULATION OF AMINO ACIDS BY STRAIN NO. 29-4 AND β IM4

Strain No. 29-4 and β IM4 were cultured at 30°C in ET-II medium for 72 hr. Amino acids were assayed with an amino acid analyser.

Strain	Amino acids produced (g/liter) ^a						
	Thr	Lys	Pro	Glu ^b	Ile	Asp	Phe
β IM4	3.82	0.31	0.25	0.20	0.14	0.09	0.06
29-4	13.40	0.03	0.00	0.13	0.12	0.00	0.02

^a The amounts of amino acids other than those represented here were less than 0.05 g/liter in both strains.

^b Or Homoserine.

TABLE II. DETERMINATION OF PLASMID COPY NUMBER

Plasmid	Molecular size	ccc Plasmid DNA (% of total DNA)	Copy number per chromosome ^a
pBR322	4.4 kb	2.7%	26
pAJ294	13.0 kb	3.4%	11

^a The molecular size of the chromosome was estimated to be 4,200 kb from the data of Cairns.¹⁸⁾

FIG. 4. Restriction Map of pAJ294.

B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I. ap, ampicillin resistance gene; tc, tetracycline resistance gene; thr, threonine gene. The double line represents the pBR322 genome.

amino acids other than threonine decreased markedly in the case of No. 29-4 as compared with those in β IM4.

Restriction mapping of the pBR322-thr recombinant plasmid

The recombinant plasmid obtained from strain No. 29-4, named pAJ294, was digested with various restriction enzymes, and the patterns of DNA fragmentation were analysed by agarose gel electrophoresis. Thus, the restriction map of pAJ294 was established as shown in Fig. 4.

Genetic analysis of threonine genes cloned in recombinant plasmid pAJ294

The threonine biosynthetic enzymes are coded by three structural genes; *thrA*, *thrB* and *thrC*, which are organized as an operon. To determine which genes of the operon were

cloned, recombinant plasmid pAJ294 was analysed genetically. Strains No. 1 (*thrA*), No. 43 (*thrB*) and No. 313 (*thrC*) were transformed with pAJ294 to Amp^r, and each 10 Amp^r transformants were tested for threonine requirement. All of them were Thr⁺. This result shows that all three genes of the threonine operon were cloned simultaneously in pAJ294.

Copy number of pAJ294

The copy number of pAJ294 was determined by the CsCl-EtBr density gradient centrifugation method as described in MATERIALS AND METHODS. The results are shown in Table II.

Homoserine dehydrogenase activity

In order to determine whether the level of the threonine biosynthetic enzymes increased in the recombinant strain, No. 29-4 and β IM4 were cultured in L medium, and crude extracts were prepared by sonication. The homoserine dehydrogenase activity in the extract of No. 29-4 (4.3 nmol/min/mg of protein) was about 5 times higher than that of β IM4 (0.8 nmol/min/mg of protein). This would be a result of the gene dosage effect.

DISCUSSION

In this paper we have described the remarkable improvement of L-threonine producing strains of *E. coli* using recombinant DNA techniques. Our experiments have demonstrated that the recombinant DNA techniques are very effective for the overproduction of amino acids, as previously shown by Debatov *et al.*¹⁴⁾ for L-threonine, Aiba *et al.*¹⁵⁾ for L-tryptophan, and Le Reverend *et al.*¹⁶⁾ for L-

lysine.

Although our results show a clear positive effect of gene amplification on the level of the biosynthetic enzymes and the productivity of threonine, the level of homoserine dehydrogenase activity in recombinant strain No. 29-4 was rather low (5 times higher than β IM4) as compared with the copy number of the threonine genes (1 copy on the chromosome and 11 copies on the plasmids). There might be two possible explanations for the observation. (1) As the attenuator region in the threonine operon we cloned would remain unchanged, expression of the genes might be repressed under the conditions we used. It is reasonable to say that the attenuator works more effectively in the recombinant strain than the parental strain, because the former must have a higher threonine level than the latter. (2) It might be possible that expression of the threonine operon on the plasmid would be insufficient as compared with that on the chromosome. Some mutation in the promoter region or excision of some important DNA sequence in the upstream region by *Hind*III digestion might occur and affect expression in the threonine operon on the plasmid. Cossart *et al.*¹⁷⁾ also discussed the possibility that promoter-down mutation occurred on the threonine operon they cloned. We observed that No. 29-4 (pAJ294 in β IM4) showed much higher productivity than No. 29 (pAJ-294 in β IM4 Thr⁻). This could also be explained by insufficient expression of the gene on the plasmid.

As shown in Fig. 4, the cloned threonine operon is on the *Hind*III fragment, about 9 kb in size. From the results of other workers,^{19,20)} the coding region of the *thrA* gene starts at 514 bp from the upper *Hind*III site, followed by *thrB* and *thrC* in the clockwise direction on the plasmid map. The *Eco*RI site in the 9 kb fragment lies in the *thrC* gene.^{17,20)} The threonine operon may not extend beyond the *Bam*HI site in the fragment, because we could obtain a smaller plasmid (about 10.5 kb) containing the whole threonine operon by circularizing the larger *Bam*HI-generated fragment

of pAJ294. By the way, cutting of the smaller *Bam*HI fragment off from pAJ294 had no significant effect on the production of L-threonine (data not shown).

We assume that the specifically elevated level of threonine biosynthetic enzyme activities in the recombinant strain must have caused the high level of L-threonine accumulation and less by-production of other amino acids. These characteristics are very advantageous for isolation and purification of the amino acid from culture broth. From the aspect of practical application, stability of the plasmid is also very important. We will describe experimental results relating to this problem in the following paper.

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