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Application of the tryptophanase promoter to high expression of the tryptophan synthase gene in *Escherichia coli*

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Summary. The application of an inducible regulation system using the tryptophanase operon promoter (TPase promoter; P_{tna}) was examined for its high expression of the tryptophan synthase (TS) gene in *Escherichia coli*. The main problem in the application of P_{tna} for industrial purposes is catabolite repression by glucose, since glucose is the most abundant carbon source. However, this problem could be avoided by changing glucose to an organic acid, such as succinate, fumarate, malate and acetate, in the course of cultivation after glucose initially added was completely consumed. Under these conditions, L-tryptophan was also used to induce tryptophan synthase. Thus, the specific activity of TS in *E. coli* strain no. 168 harbouring pBR322F-P_{tna}TS was increased 500-fold compared to that of the cultured host strain. About 1 mol L-tryptophan/l reaction mixture was formed from indole and L-serine at 37° C for 3.5 h.

Introduction

Plasmids can serve as powerful tools for enhancing the synthesis of a specific enzyme and/or metabolite in the cultivation of microorganisms, particularly when combined with gene manipulation techniques. If the microbial production of useful compounds by gene manipulation techniques is to be considered for industrial purposes, it is necessary to establish the following methods: (1) stabilization of the recombinant plasmid; (2) high expression of the structural gene; (3) high density cultivation. However, there is little information available on regulation of the promoter and over-production of gene products for industrial purposes (Kawai et al. 1986; Matsui et al. 1990). Until now, for the expression of a foreign structural gene, the *trp* promoter has been widely used since it is strong and inducible.

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We reported earlier that stability of the recombinant plasmid-encoded tryptophan synthase (TS) gene and high expression of the gene could be established by application of mini-F DNA and the *trp* promoter (Yukawa et al. 1985, 1987, 1988). In this case, the TS gene is expressed at a high level by addition of 3-indoleacrylic acid (IA) to the culture medium. However, it might be better to avoid the use of IA for the following reasons: (1) bacterial growth is strongly inhibited by IA addition; (2) it is relatively difficult to optimize the timing and the amount of IA addition in large-scale cultivation (Yukawa et al. 1987); (3) the cost of IA is high; (4) possible contamination of IA in the final product. In the past, some inducible promoters that function in *Escherichia coli* have been reported. Among these promoters, we examined the tryptophanase (TPase) promoter for expression of a foreign structural gene to avoid the problems with IA. The TPase promoter is reported to be markedly repressed by glucose (Freundlich and Lichstein 1960; Botsford and DeMoss 1971; Deeley and Yanofsky 1981). Due to this problem with glucose, the TPase promoter has not been practical for industrial purposes. Therefore, very little is known about high expression of foreign structural genes using the TPase promoter. Hence, we evaluated the industrial application of the TPase promoter by using the TS gene as a foreign structural gene. It was found that high expression from the TPase promoter can be established by changing the carbon source from glucose to an organic acid.

In this report, we studied the regulation of gene expression by the TPase promoter and high density cultivation of *E. coli* harbouring the mini-F plasmid.

Materials and methods

Bacterial strains and plasmids. The bacterial strain used as host was strain no. 168 (*tnaA*, F^-, λ^-) isolated from *E. coli* K-12 ATCC 27325 (F^-, λ^-) by N-methyl-N'-nitro-N-nitrosoguanidine treatment. A TnaA mutant was required in this study to avoid formation of tryptophan by TPase produced from chromosomal *tnaA*. The TnaA mutant, strain no. 168, was selected as a negative

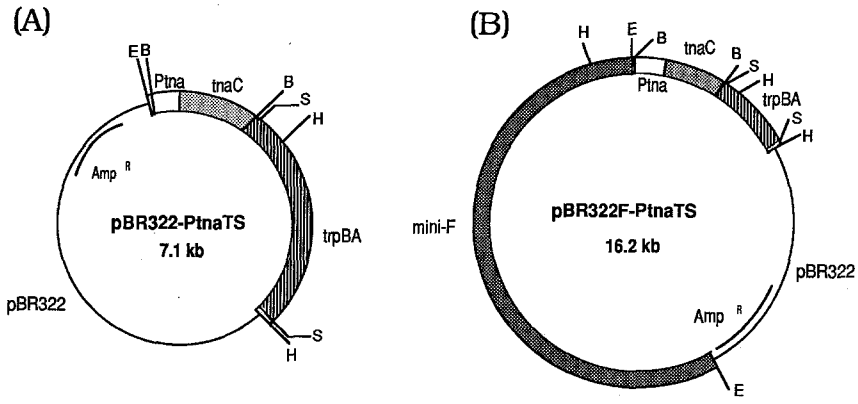

Fig. 1. Restriction maps of plasmids: *B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *S*, *Sal*I

producer of indole from tryptophan by the method of Wood et al. (1947).

The following plasmid was constructed: pBR322-P_{tna}TS [TPase promoter-*tnaC* + *trpBA* + pBR322], pBR322F-P_{tna}TS [TPase promoter-*tnaC* + *trpBA* + pBR322 + 9.1-kb mini-F fragment (*Eco*RI-*Eco*RI)].

Construction of plasmids. The 2.4-kb *Hin*cII fragment containing the *trpBA* gene from plasmid pBR322-*trp* (Yukawa et al. 1985) was ligated with *Sal*I linker and digested with *Sal*I. This DNA fragment was inserted into the *Sal*I site of the plasmid pUC119 (named pUC119-TS). The *E. coli* tryptophanase operon was initially cloned by the method of Deeley and Yanofsky (1982) and the tryptophanase operon subcloned into the *Bam*HI and *Hin*dIII sites of pBR322 (named pBR322-TN). The *Alu*I-*Rsa*I promoter region (0.6 kb, containing the catabolite sensitive region, −35 and −10 of the promoter, *tnaC* region and the 5′-42 bp of *tnaA*) from pBR322-TN was ligated with the *Bam*HI linker and digested with *Bam*HI. This DNA fragment was inserted into the *Bam*HI site of the plasmid pUC119-TS (named pUC119-P_{tna}TS) in front of the *trpBA* gene.

The 3.1-kb *Hin*dIII-*Eco*RI (partially digested by *Hin*dIII) fragment of pUC119-P_{tna}TS, spanning from the TPase promoter region to the TS structural gene, was subcloned into the pBR322 digested with *Hin*dIII and *Eco*RI to form pBR322-P_{tna}TS (Fig. 1A). Finally, the 9.1-kb *Eco*RI fragment responsible for partitioning the F-plasmid was inserted into the *Eco*RI of pBR322P_{tna}TS to form pBR322F-P_{tna}TS (Fig. 1B).

Media. The nutrient medium contained (g/l): $(NH_4)_2SO_4$, 3; KH_2PO_4, 1.6; K_2HPO_4, 5.5; $MgSO_4 \cdot 7H_2O$, 0.4; $FeSO_4 \cdot 7H_2O$, 0.1; yeast extract, 1; peptone, 1; in distilled water, pH 7.2.

The medium for plates contained Luria-Bertani (LB) medium and 2% (w/v) agar.

Culture conditions. The seed culture was prepared as follows. A loopful of the micro-organisms from a stock culture was inoculated into 100 ml medium (glucose concentration, 4 g/l) in a 500-ml shake flask and then cultured on a rotary shaker at 37° C for 15 or 24 h.

The seed culture (30 ml) was transferred into a fermentor (working volume, 1.5 l, Type MBC, Able, Tokyo, Japan). The initial concentration of glucose in the culture medium was 60 g/l. The temperature was controlled at 37° C and the pH at 7.2 by addition of NH_4OH solution (containing 25% NH_3). Aeration and agitation were operated at 1.0–1.5 vvm and 600–1000 rpm, respectively. L-Tryptophan powder was supplied to the culture as required.

Analysis. Cell growth was measured as grams wet weight of cells harvested from 1 l broth by centrifugation (10,000*g*, 15 minutes).

Enzyme assay. Preparation of crude extract was carried out as follows. Cells harbouring plasmid were cultured in nutrient medium. Harvested cells were washed with 100 mM TRIS-HCl (pH 7.8) and frozen overnight at −20° C. Frozen cells (200 mg) were resuspended in 1 ml of 100 mM TRIS-HCl (pH 7.8) and sonicated for 3 min (sonifier: model 200, Branson Sonic Power, Danbury CT, USA) at 10 kHz in ice/water. Cell debris was removed by centrifugation at 12,000 rpm for 40 min and the supernatant was used as crude extract. The TS assay with indole and DL-serine as substrates was performed according to Smith and Yanofsky (1962), and the β-lactamase assay according to Sargent (1968). The protein concentration was measured by the method of Bradford (1976). One unit of activity is defined as the amount of enzyme that gives rise to the formation of 0.1 μmol tryptophan in 20 min at 37° C. Specific activity is expressed as the number of units per milligram of protein.

Phenotypic stability of plasmid. E. coli cells harbouring the plasmids, which were grown in LB medium containing 50 μg/ml ampicillin (Amp) to the stationary phase, were inoculated into LB medium at approx. 50 cells/100 ml, grown at 37° C for the average generations indicated, and then aliquots of the cultures were diluted and spread on LB plates. After overnight incubation at 37° C, 100 colonies from each plate were examined for their phenotypes by transferring them with toothpicks to selective plates (LB + Amp).

Formation of tryptophan by the cell harbouring pBR322F-P_{tna}TS. The reaction mixture contained: indole initially 200 mM, L-serine, 1.0 M; pyridoxal 5′-phosphate, 40 μM; triton X-100, 10% (w/v) in 100 mM sodium phosphate buffer (pH 8.0). Indole powder was supplied to the mixture intermittently to a final concentration of 1.0 M. Twenty grams (frozen base) of intact cells were transferred to 1 l reaction mixture in a 3-l fermentor and indubated at 37° C. Samples were diluted with distilled water and the L-tryptophan and L-serine contents were measured by HPLC (model LC-5A, Shimadzu, Tokyo, Japan).

Purification of tryptophan. The tryptophan accumulating as a precipitate in the reaction mixture was isolated and purified according to Nakazawa et al. (1972). The optical activity of tryptophan was analysed by a polarimeter (model DIP-380, Japan Spectroscopic, Tokyo, Japan).

Chemicals. Yeast extract and peptone were purchased from Daigo Nutritive Chemicals, Osaka, Japan. The other reagents were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Results and discussion

Stability of mini-F recombinant plasmids

Stabilization of recombinant plasmids by the mini-F fragment has been reported previously (Yukawa et al.

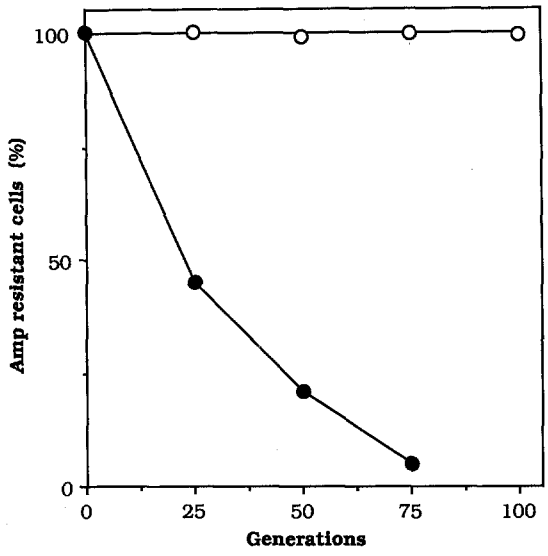

Fig. 2. Phenotypic stability of plasmids: ●, pBR322-P_{tna}TS; ○, pBR322F-P_{tna}TS. Amp, ampicillin

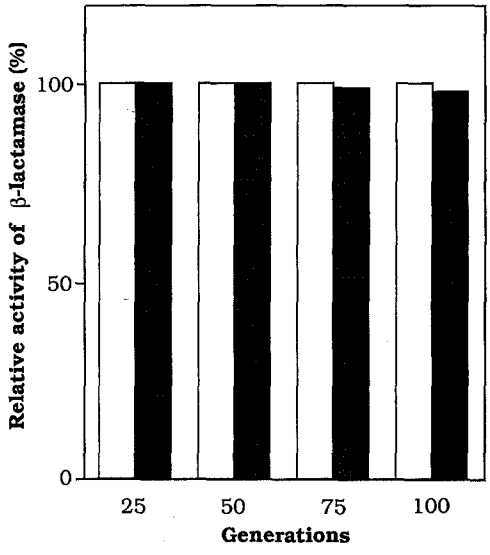

Fig. 3. β-Lactamase activity in strain no. 168 harbouring pBR322F-P_{tna}TS under selective and non-selective conditions: □, cells grown in Luria-Bertani (LB) medium + Amp (50 μg/ml); ■, cells grown in LB medium alone

1985, 1987, 1988). The stability of the TS-gene-bearing mini-F plasmid employed in this study was also tested. As shown in Fig. 2, pBR322-P_{tna}TS (without mini-F DNA) was unstable, as evidenced by loss of about 75% of the cells after 50 generations of growth in LB. However, pBR322F-P_{tna}TS (with mini-F DNA) was stably maintained in strain no. 168.

We examined the change in the plasmid copy number to determine whether this stability depended on a decrease in plasmid copy number. In terms of the plasmid copy number, a linear correlation has been reported between the production of β-lactamase and the number of gene copies (Ogura et al. 1980). As shown in Fig. 3, the β-lactamase activity of the cells harbouring pBR322F-P_{tna}TS was the same under selective or non-selective conditions, indicating that the copy number of both plasmid was equivalent. The activity of the cells harbouring pBR322F-P_{tna}TS was the same as that of the cells harbouring pBR322 under selective conditions (data not shown). Therefore, the copy number of pBR322F-P_{tna}TS was estimated to be the same as pBR322 in strain no. 168.

Regulation of gene expression

In the *E. coli* tryptophanase operon, expression of the tryptophanase gene is inducible by L-tryptophan and is subject to catabolite repression control, i.e., cyclic AMP (cAMP) and the catabolite gene activator protein (CAP) are required (Ward and Yudkin 1976). The localization of the promoter and CAP site controlling TPase gene expression indicates that transcription initiation at the TPase promoter involves cAMP-dependent, CAP-facilitated binding of RNA polymerase to the DNA (Deeley and Yanofsky 1982). For application of the TPase promoter, therefore, it is necessary to control glucose repression and the induction by L-tryptophan.

To avoid glucose repression for the expression of the TS gene, carbon sources other than glucose were tested in shake-flask cultivations. As shown in Table 1, it was found that in the presence of carbon sources other than glucose, the specific activity of TS was higher. However, the growth rate was much lower than in glucose-grown cultures. From these results, we concluded that derepression of the TPase promoter is possible by separation of the phase of gene expression from the growth phase using both glucose and other carbon source.

To determine the effective conditions for induction, the L-tryptophan concentration required for TS gene expression was examined. As shown in Table 2, when 300 μg/ml L-tryptophan was added to the nutrient medium, the specific activity of TS was increased fivefold. The residual L-tryptophan at the end of cultivation was about 0.2 g/l. Therefore, it was important to maintain

Table 1. Effect of carbon sources on tryptophan synthase (TS) expression

Carbon source	Growth[a] (%)	Specific activity[b] of TS (%)
Succinate	26	100
Fumarate	18	98
L-Malate	28	97
Acetate	30	95
Glucose	100	3

Strain no. 168 harbouring pBR322F-P_{tna}TS was cultivated with nutrient medium containing 0.5 g/l of L-tryptophan for 8 h in a 500-ml shake flask

[a] The value of growth obtained with glucose is taken as 100%

[b] The specific activity value obtained from cells grown on succinate is taken as 100%

Table 2. Effect of L-tryptophan concentration on tryptophan synthase (TS) expression

L-Tryptophan concentration (%)	Growth (%)	Specific activity of TS (%)
0	127	20
0.005	127	40
0.01	118	60
0.03	100	100
0.05	100	100

Strain no. 168 harbouring pBR322F-P_{tna}TS was cultivated with nutrient medium containing 0.2% succinate for 8 h in a 500-ml shake flask. The value of growth and specific TS activity obtained at 0.05% L-tryptophan were taken as 100%

the L-tryptophan concentration in the culture medium at 0.2 g/l or higher for effective induction.

Regulation of gene expression in fed-batch cultivation

Our results of shake-flask cultivations indicate that the positive regulation of the TPase promoter is possible by separating the phase of gene expression from the growth phase.

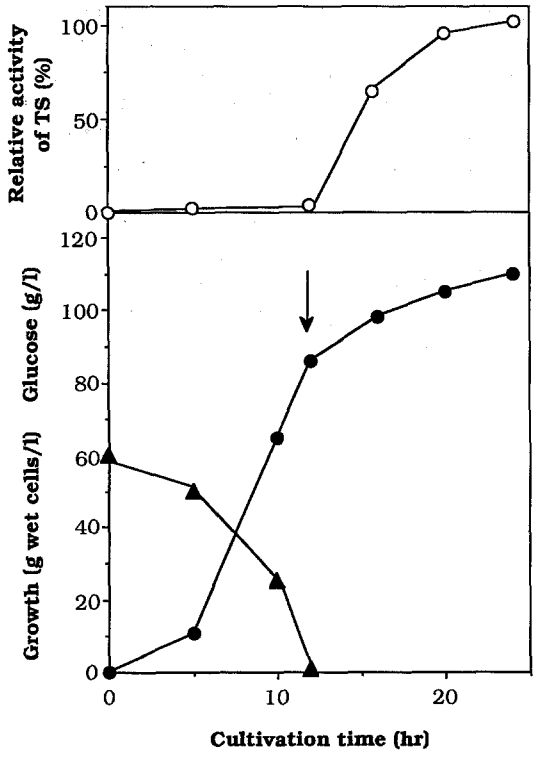

Fig. 4. Time course of tryptophan synthase (TS) gene expression in strain no. 168 harbouring pBR322F-P_{tna}TS by changing carbon sources: ●, biomass concentration: ▲, glucose concentration; ○, relative activity of TS obtained after 24 h cultivation was taken as 100%. The *arrow* indicates the addition of L-tryptophan (1.0 g/l) as an inducer and succinate (10 g/l) as a carbon source after consumption of glucose

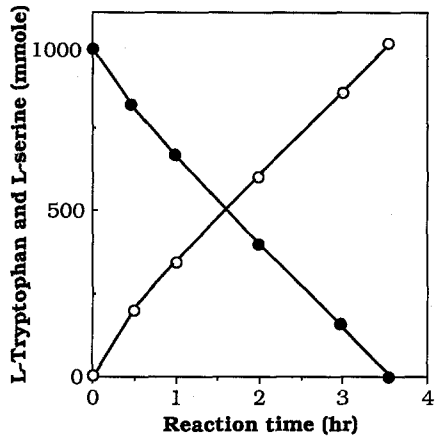

Fig. 5. Formation of L-tryptophan from indole and L-serine by strain no. 168 harbouring pBR322F-P_{tna}TS: ○, L-tryptophan; ●, L-serine

Figure 4 shows the results of derepression of the promoter in the TPase operon by replacing glucose with another carbon source. For the first 12 h, the fed-batch culture was supplied with glucose and consequently TS formation was markedly repressed. When the glucose was completely consumed, succinate was supplied to induce expression of the TS gene. The specific activity of TS increased by 500-fold compared to the host strain cultured under the same conditions, and reached the maximum value (690 units/mg protein) within 5 h, of initiating succinate feeding. The final cell concentration was about 120 g wet cells/l. By this feeding change, a relatively high growth rate of the recombinant ($\mu_{max} = 0.27\ h^{-1}$) was obtained with efficient expression of the TS gene. These methods may be applicable for expression of other genes with the promoter of the TPase operon.

Formation of L-tryptophan from indole and L-serine

The enzymatic formation of L-tryptophan from indole and L-serine was carried out using the cells harbouring pBR322F-P_{tna}TS cultured in Fig. 4. The cells were frozen at −20° C for 2 days in order to suppress enzymatic degradation of L-serine and to enhance the permeability of substrates into the cell. As shown in Fig. 5, about 1 mol L-tryptophan/l was formed at 37° C for 3.5 h. The crystals were identical to authentic L-tryptophan with respect to their optical activity $[\alpha]_D^{20} - 31.5°$ (c = 1 in H_2O).

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