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Stabilization of an *E. coli* plasmid by a mini-F fragment of DNA

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SUMMARY

In an *Escherichia coli* K-12 strain (*trpA trpE tnaA*) cultured in LB broth without selective pressure, a pBR322 derivative containing the gene for tryptophan synthase (pBR322-*trpBA*) was found to be unstable. After 70 cell-number doublings, only 50% of the host cells retained the gene for ampicillin resistance (Ap^r). Insertion of the mini-F fragment of F factor DNA into this plasmid could effectively reduce the plasmid loss. Partial derepression of the tryptophan promotor-operator by 3-indoleacrylic acid further decreased the stability of the pBR322-*trpBA* but not that of the mini-F inserted plasmid (pBR322F-*trpBA*). The vector pBR322F-*trpBA* could be maintained at high copy number in the culture after 100 generations of growth; the culture was able to overproduce tryptophan synthase in the presence of 3-indoleacrylic acid. L-Tryptophan was produced from indole and L-serine using an *E. coli* host transformed with pBR322F-*trpBA* DNA. After 8 h of incubation, the expression level was approximately 180 g/l.

INTRODUCTION

L-Tryptophan, an essential amino acid, is generally deficient in cereal protein. Because the quality of cereal protein can be improved by the addition of L-tryptophan, a large demand is anticipated for this amino acid as a feedstuff and foodstuff.

Many studies have been published on microbial production of L-tryptophan and these methods may be summarized into two types: fermentative and enzymatic production.

From the standpoint of industrial L-tryptophan production, enzymatic production is generally simpler than fermentative production. However, the enzyme is too costly to use in commercial processes. As an alternative to the enzymatic process, we constructed an *Escherichia coli* strain that produces high levels of tryptophan synthase by recombinant DNA methods.

In many industrial applications of recombinant DNA technology, the instability of a recombinant plasmid is a serious problem. Generally, to ensure stable inheritance of a recombinant plasmid, the addition of antibiotics is applied as a selective pressure. However, this method might not be practical for several reasons. For example, antibiotics add,

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considerably, to the cost of the medium. They may also upset the operation of the wastewater treatment system.

In a previous paper, we reported that an inserted mini-F fragment could stabilize an otherwise unstable pBR322-*trp* operon even in a *tnaA* mutant in the presence of 3-indoleacrylic acid [16]. In this paper, we employed a mini-F fragment to increase stability of tryptophan synthase gene-bearing plasmids in *E. coli* and investigated the feasibility of this process for L-tryptophan production from the precursors indole and L-serine.

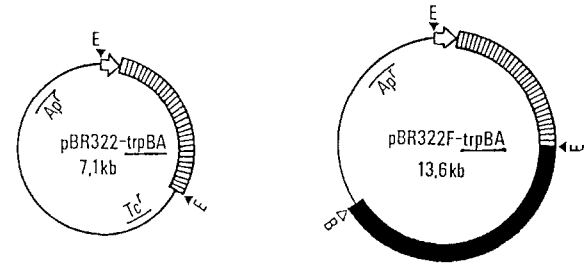

Fig. 1. Filled region, the mini-F segment; hatched region, segment including *trpB* and *trpA*; open region, tryptophan promotor-operator segment; fine line, pBR322-derived segment. E and B represent the restriction endonuclease *Eco*RI and *Bam*HI sites, respectively.

MATERIALS AND METHODS

Materials

Ampicillin (Ap), tetracycline (Tc) and 3-indoleacrylic acid (IA) were purchased from Sigma, St. Louis, MO, U.S.A. Restriction enzymes and DNA ligase were from Takara Shuzo Co., Ltd., Kyoto, Japan. All other reagents were from Wako Pure Chemical Industries Ltd., Osaka, Japan.

Bacterial strains

E. coli K-12 strain JK-268 (F⁻ *trpE*61 *trpA*62 *tnaA*-5 *purB*58 *dadR*1, *E. coli* Genetic Stock Center, Yale University) was used as the host for plasmids.

Media and growth conditions

Cells were cultured either in minimal medium MM [15] (containing 0.2% glucose and supplemented with 50 μg adenine per ml) or broth medium LB [2] (supplemented with 50 μg adenine per ml and 0.1% glucose) at 37°C with constant shaking. Plates contained 1.5% agar. Ap was added to selective plates at a concentration of 50 μg per ml.

DNA transformation, isolation and manipulation

Transformation of cells followed the procedure of Cohen et al. [3]. Plasmid DNA was prepared by the cleared lysate method [4] and by CsCl-ethidium bromide density gradient centrifugation [5].

Plasmid construction

Plasmids were constructed as depicted in Fig. 1.

Tryptophan promotor-operator DNA fragment was obtained by using the promoter-probe plasmid vector pKK232-8 (Pharmacia. Co, Ltd.). Tryptophan operon DNA from pBR322-*trp* [16] was digested with *Alu*I and *Rsa*I and then ligated to *Sma*I-digested pKK232-8 DNA. *E. coli* K-12 strains transformed with ligated DNA and transformants which have a chloramphenicol resistance phenotype were selected. The plasmid DNA was isolated from these transformants and tryptophan promoter-operator DNA fragment was purified by digesting the plasmid DNA with *Eco*RI and *Bam*HI. This DNA fragment was treated with S1 nuclease to produce blunt ends and was ligated to a DNA fragment containing *trpB* and *trpA* genes which was obtained by partially digesting tryptophan operon DNA of pBR322-*trp* with *Hin*cII. Furthermore. *Eco*RI linker was ligated and the resulting DNA fragments were religated to the *Eco*RI-digested pBR322 to form pBR322-*trpBA*.

To construct the pBR322F-*trpBA*, pBR322F was first constructed by inserting the mini-F fragment prepared by digesting miniF-*trp* [16] with *Eco*RI and *Bam*HI into the *Eco*RI and *Bam*HI sites of pBR322. pBR322F-*trpBA* was constructed by inserting the *Eco*RI fragment containing *trpB* and *trpA* genes prepared by digesting pBR322-*trpBA* with *Eco*RI into the *Eco*RI site of pBR322F.

The reaction conditions for restriction endonucleases and T4 DNA ligase were those reported by Maniatis et al. [6]. The molecular sizes of the con-

structed plasmids and DNA fragments were determined by electrophoresis in horizontal agarose slab gels with a buffer containing 20 mM Na-acetate, 1 mM ethylene diaminetetraacetate and 33 mM Tris-HCl (pH 7.8). The direction of insertion in each step was examined by digesting the plasmids with appropriate restriction endonucleases and then determining the sizes of the fragments formed.

Plasmid stability test

Cells of strain JK268 (*trpA trpE tnaA*) harboring the plasmids, which were grown in MM to the stationary phase, were inoculated into LB at approx. 50 cells per 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 as to their phenotypes by transferring them with toothpicks to selective plates (LB + Ap).

Agarose gel electrophoresis

The cells harboring pBR322F-*trpBA* and pBR322-*trpBA* were precultured overnight in LB + Ap, and then cultured in LB + IA (200 mg/ml) at 37°C for 100 generations. Plasmid DNA extracted from 10 mg of cells and collected by centrifugation was analyzed. Plasmid DNA was digested with *Eco*RI and electrophoresis was carried out with 0.8% agarose gel.

Enzyme assay

Tryptophan synthase activity in crude cell extracts was assayed by the method of Smith and Yanofsky [13] and β-lactamase activity by the method of Sargent [12]. The cells harboring pBR322F-*trpBA* were precultured overnight in LB + Ap (50 μg/ml) and then cultured in LB + IA (200 μg/ml) with or without Ap (50 μg/ml) at 37°C for 25, 50, 75 and 100 generations. One unit of activity was expressed as the cleavage of 1 μmol β-lactam ring per min at 30°C.

Production of L-*tryptophan*

E. coli JK-268 transformed with pBR322F-*trpBA* was grown in LB medium supplemented with 200 mg/l IA. Incubation was carried out at 37°C in a 500 ml flask containing 100 ml of medium on a rotary shaker operated at 150 rpm. Cells were harvested by centrifugation. The reaction mixture was composed of indole 5 g (final 11 g), L-serine 15 g, pyridoxal 5'-phosphate 1 mg, NaCl 10 g, Triton X-100 8 g, and 100 mM Tris-HCl (pH 7.8) 100 ml. 5 g of whole cells were transferred to 100 ml of reaction mixture in a 500 ml flask and incubated at 37°C. Indole was added to the reaction mixture intermittently.

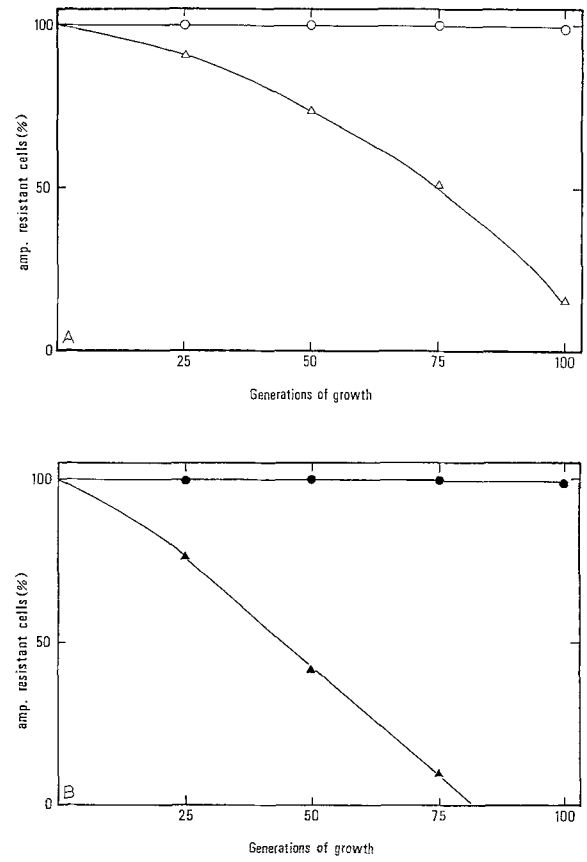

Fig. 2. (A) Stability of plasmids. The plasmid stability test is described in Materials and Methods. The cells for the stability test were obtained from 25, 50, 75 and 100 generations of growth. ○, *E. coli* JK268 harboring pBR322F-*trpBA*; △, *E coli* JK268 harboring pBR322-*trpBA*. (B) Stability of plasmids under partial derepression with IA. IA was added to LB medium at 200 μg/ml to derepress the tryptophan promoter-operator. The cells for the stability test were from the same generations as in (A). ●, *E. coli* JK268 harboring pBR322F-*trpBA*; ▲, *E. coli* JK268 harboring pBR322-*trpBA*.

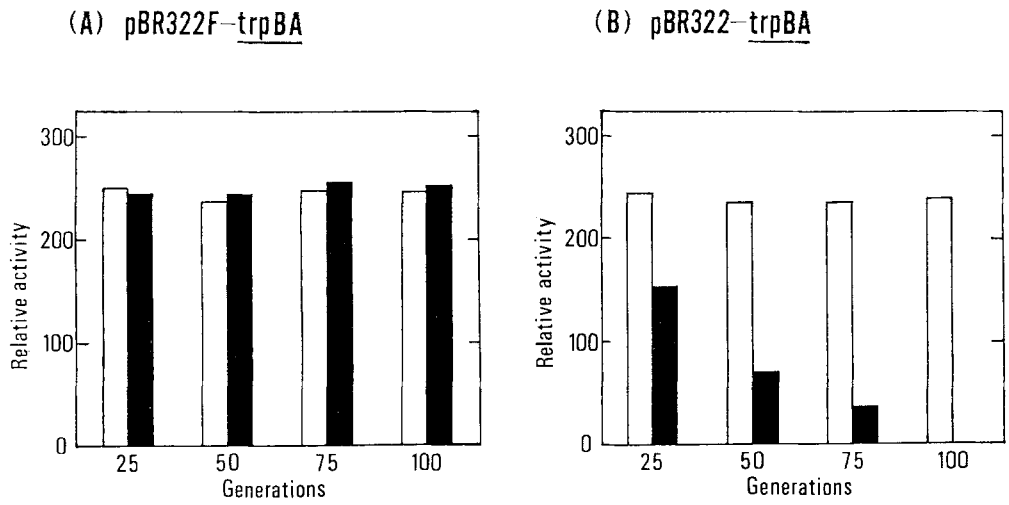

Fig. 3. Relative activities of tryptophan synthase in the cells harboring composite plasmids. The cells harboring the composite plasmids were precultured overnight in LB + Ap (50 μg/ml) and then cultured in LB + IA (200 μg/ml) with or without Ap at 37°C for 25, 50, 75 and 100 generations. The tryptophan synthase assay is described in Materials and Methods. The activity obtained in the parent strain (*trp*) was taken as 1. □, cells were grown in LB + IA (200 μg/ml) + Ap (50 μg/ml); ■, cells were grown in LB + IA (200 μg/ml).

RESULTS

Phenotypic stabilities

The plasmid pBR322-*trpBA* was unstable as evidenced by loss of about 50% in cells after 70 generations of growth in LB (Fig. 2A). Addition of IA, an efficient competitive inhibitor of the repressor, and tryptophanyl-tRNA synthetase [14] to the medium enhanced the instability (Fig. 2B). However, composite plasmid pBR322F-*trpBA* was stably maintained in strain JK 268 under nonselective conditions and also maintained stably in cells partially derepressed with IA (Fig. 2).

Tryptophan synthase and β-lactamase level

As shown in Fig. 3, specific activities of the cells harboring pBR322F-*trpBA* were maintained at a high level under selective and nonselective conditions. However, those of the cells harboring pBR322-*trpBA* were also maintained at a high value under selective conditions, but decreased rapidly under nonselective conditons. This decrease almost coincided with the phenotypic stability of pBR322-*trpBA* plasmids under nonselective conditions.

Plasmids derived from ColE1 are normally present at about 20 copies per bacterial chromosome, and a linear correlation has been reported between the production of β-lactamase and the number of gene copies [11].

As shown in Fig. 4, β-lactamase activity of the cells harboring pBR322F-*trpBA* was maintained at the same level under selective and nonselective con-

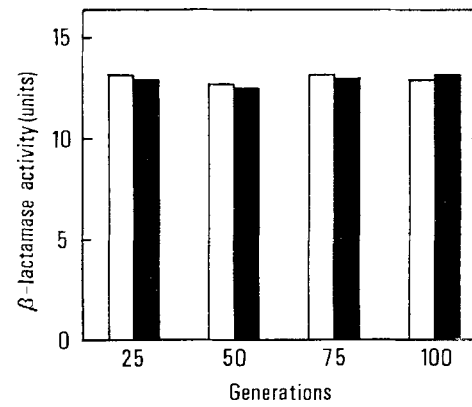

Fig. 4. β-Lactamase activities in the cells harboring composite plasmid under selective and nonselective conditions. □, cells were grown in LB + IA (200 μg/ml), + Ap (50 μg/ml); ■, cells were grown in LB + IA (200 μg/ml).

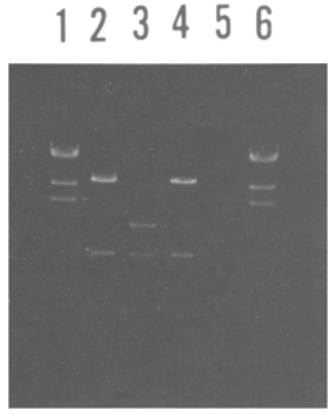

Fig. 5. Agarose gel electrophoresis patterns of pBR322F-*trpBA* and pBR322-*trpBA* plasmid digested with *Eco*RI. Lanes 1 and 6, *Hin*dIII-digested λ phage DNA as molecular size marker; lane 2, pBR322F-*trpBA* in preculture; lane 3, pBR322-*trpBA* in preculture; lane 4, pBR322F-*trpBA* after 100 generations in main culture; lane 5, pBR322-*trpBA* after 100 generations in main culture.

ditions. This result implies that the number of copies was the same for these cultural conditions. As shown in Fig. 5, the variance of molecular size and DNA level of pBR322F-*trpBA* after 100 cell-number doublings was not observed, but no DNA level was detected after 100 generations in pBR322-*trpBA*.

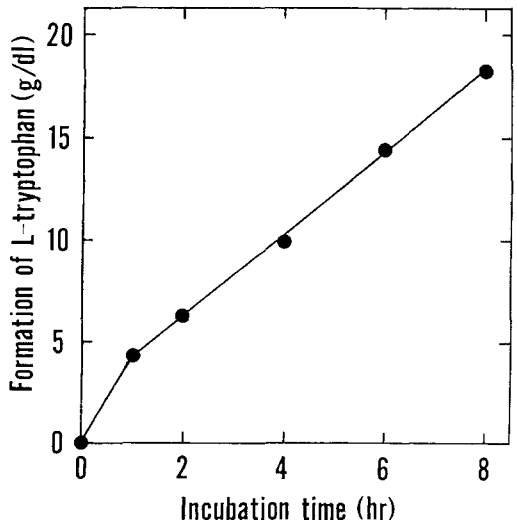

Fig. 6. Time course of formation of L-tryptophan from indole and L-serine.

The production of L-tryptophan from indole and L-serine was studied using bacterial cells harboring pBR322F-*trpBA*.

To release the inhibition of tryptophan synthase by indole, Triton X-100 was added to a reaction mixture as a reservoir of indole. After 8 h incubation, 180 g/l of L-tryptophan was formed as shown in Fig. 6.

DISCUSSION

For industrial purposes, genetic stability of plasmid-bearing strains is of importance. For low copy number plasmids, such as F [9], pSC101 [7] and P1 [1], special partitioning functions have been identified that ensure that each daughter cell receives at least one plasmid during cell division. For high copy number plasmids, such as ColE1, it is generally believed that partitioning is achieved by random distribution of plasmids, and high copy number plasmids are often unstable.

In this report, we describe efforts to stabilize a plasmid construction by insertion of a mini-F fragment of DNA.

The pBR322F-*trpBA* was maintained at high copy number in *E. coli* after 100 generations and tryptophan synthase was overproduced in the presence of 3-indoleacrylic acid.

In the mini-F plasmid, two distinct regions have been shown to confer the stability: a 3.0 kb region including the *sopA*, *sopB* and *sopC* genes that most likely specify the partitioning [9], and the 0.7 kb *ccd* region that couples host cell division to plasmid proliferation [10]. Miki et al. [8] also obtained results consistent with these theories. Hiraga et al. proposed a model for the partition mechanism of the mini-F plasmid, a low copy number plasmid. The SopC region acts as a specific DNA site necessary for the actual partitioning, interacting with the cellular components consisting of SopB protein indirectly with the SopC region, and/or SopA protein may regulate the expression of the SopB gene. The expression of the SopA gene may be regulated by

SopB protein. The hypothetical partition apparatus (SopC binding site) is assumed to be related to the cell membrane [9].

Our observation that the mini-F functioned in stabilizing a high copy number plasmid cannot be explained sufficiently by the partition mechanism. The fact that pBR322F-*trpBA* was stably kept at a high copy number in *E. coli* indicates that the intensity of replicon on pBR322 is superior to that on mini-F DNA. As a possible mechanism, when one copy of mini-F plasmid is inherited at least to a daughter cell in cell division, the copy number of that plasmid may increase to high copy by replicon on pBR322 in daughter cell.

The present results indicate that this host-plasmid system is a feasible and practical method for enzymatic synthesis of L-tryptophan from indole and L-serine. After 8 h incubation, expression levels of L-tryptophan were estimated at 180 g/l.

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REFERENCES

1 Austin, S. and A. Abeles. 1983. Partition of unitcopy miniplasmids to daughter cells. II. The partition region of miniplasmid P1 encodes an essential protein and a centromere-like site at which it acts. J. Mol. Biol. 169: 373–387.

2 Bertani, G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J. Bacteriol. 62: 293–300.

3 Cohen, S.N., A.C.Y. Chang and L. Hsu. 1972. Nonchromosomal antibiotic resistance in bacteria; genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA 69: 2110–2114.

4 Godson, G.N. and D. Vapnek. 1973. A simple method of preparing large amounts of φX174 RF1 supercoiled DNA. Biochim. Biophys. Acta 299: 516–522.

5 Kupersztoch, Y.M. and D.R. Helinski. 1973. A catenate molecule as an intermediate in *Escherichia coli*. Biochem. Biophys. Res. Commun. 54: 1451–1459.

6 Maniatis, T., E.F. Fritsch and J. Sambrook. 1982. Molecular Cloning. Cold Spring Harbor Laboratory, p. 76, Cold Spring Harbor, New York.

7 Meacock, P.A. and S.N. Cohen. 1980. Partitioning of bacterial plasmids during cell division: a cis acting locus that accomplishes stable plasmid inheritance. Cell 20: 529–542.

8 Miki, T., K. Yoshioka and T. Horiuchi. 1984. Control of cell division by sex factor in *Escherichia coli*. I. The 42.84–43.6 F segment couples cell division of the host bacteria with replication of plasmid DNA. J. Mol. Biol. 174: 605–625.

9 Ogura, T. and S. Hiraga. 1983. Partition mechanism of F plasmid: two plasmid gene-encoded products and a cis-acting region are involved in partition. Cell 32: 351–360.

10 Ogura, T. and S. Hiraga. 1983. Mini-F plasmid genes that couple host cell division to plasmid proliferation. Proc. Natl. Acad. Sci. USA 80: 4784–4788.

11 Ogura, T., T. Miki and S. Hiraga. 1980. Copy-number mutants of the plasmid carrying the replication origin of the *Escherichia coli* chromosome: Evidence for a control region of replication. Proc. Natl. Acad. Sci. USA 77: 3993–3997.

12 Sargent, M.G. 1968. Rapid fixed-time assay for penicillinase. J. Bacteriol. 95: 1493–1494.

13 Smith, D.H. and C. Yanofsky. 1962. Enzymes involved in the biosynthesis of tryptophan. Methods Enzymol. 5: 794–806.

14 Squire, C.L., F.D. Lee and C. Yanofsky. 1975. Interaction of the *trp* repressor and RNA polymerase with the *trp* operon. J. Mol. Biol. 92: 92–98.

15 Vogel, H.J. and D. M. Bonner. 1956. Acetylornithinase of *Escherichia coli;* partial purification and some properties. J. Biol. Chem. 218: 97–104.

16 Yukawa, H., Y. Kurusu, M. Shimazu, M. Terasawa, A. Ohta and I. Shibuya. 1985. Stabilization by the mini-F fragment of a pBR322 derivative bearing the tryptophan operon in *Escherichia coli*. Agric. Biol. Chem. 49: 3619–3622.