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Extracellular Production of Alpha-Interferon by Recombinant *Escherichia coli:* PartⅣ. Effects of Ampicillin and an Inducer on the Production of Alpha-Interferon and Plasmid Stability

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유전자 재조합 대장균을 사용한 Alpha-interferon의 생산과 분비; 제4부. Ampicillin및 Inducer의 Alpha-interferon의 생산과 Plasmid 안정성에 미치는 영향

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ABSTRACT

We studied the production and excretion of alpha-interferon in recombinant *Escherichia coli* harboring plasmid pIF-Ⅲ-B, which carries alpha-interferon gene under the control of lipoprotein and *lac*UV5 promoter, and *lac* operator. Basically, the effects of concentrations of ampicillin and an inducer, IPTG, for the expression of the cloned gene, on the production of alpha-interferon and plasmid stability were studied.

The highest production of alpha-interferon was observed at 50 mg/l of ampicillin concentration and 0.5 mM of IPTG. The plasmid pIF-Ⅲ-B was maintained very stably in medium with ampicillin but segregated rapidly in medium without ampicillin. Also, the plasmid was segregated more rapidly in medium with an inducer higher than 0.5 mM.

INTRODUCTION

The hyperproduction of many useful pharmaceutical proteins in *Escherichia coli* has been made possible through the recombinant DNA technology. For the successful product formation from the fermentation system of the recombinant cells, both efficient expression of cloned genes and stable maintenance of recombinant plasmid are very important, and accumulation of the cloned gene products should not act as a severe burden on host cell growth, which sometimes are very deleterious to host cells and leads them even to death.

We previously reported the construction of recombinant plasmids pIF-Ⅲ-B and pIF-Ⅲ-C for the production and excretion of alpha-interferon in *Escherichia coli* system, which carry alpha-interferon (alpha-IFN) gene under the control of *lpp-lac* UV5 dual promoter, and *lac* operator (1). Also, the growth behaviors of the recombinant cells (2) and gene product formation in various host strains (3) were studied. Excretion of cloned gene products to

extracellular environment of recombinant cells seems to give several advantages: reduction of a possible burden of accumulation of toxic foreign gene products imposed on host strain, avoidance of products from proteolytic attack, prevention of aggregate formation, and ease in product recovery.

There are a lot of reports on the production of many useful pharmaceutical proteins using recombinant *E. coli* systems with high cell density in defined media. Usually, inorganic nitrogen source is used for the production of the recombinant proteins because organic nitrogen has reportedly bad effects on the production of the recombinant proteins in *E. coli* system(6). But, we used LB medium for the basic study of alpha-interferon production and plasmid stability for the convenience of experiments.

One of the important factors in scale-up of a fermentation of recombinant microorganisms is the instability of the plasmid employed. Plasmid instability can be divided into two categories, *i. e.*, segregational instability and structural instability. Segregational instability is defined as the loss of the complete plasmid due to defective partitioning during cell division. Structural instability is defined as a change in plasmid structure due to insertion, deletion or rearrangement of DNA, which can result in the loss of a desired gene function.

In the current report, we have described the effects of concentration of ampicillin and an inducer, IPTG on the production of alpha-IFN in relation with the maintenance of plasmid pIF-Ⅲ-B and expression of the cloned alpha-IFN gene. The inducer, IPTG, counteracts the repressors, which are produced from *lacI* gene in plasmid pIF-Ⅲ-B and bind to *lac* operator preventing the expression of alpha-IFN gene. Because the structural instability of the plasmid pIF-Ⅲ-B was not observed, the word "plasmid instability" means segregational instability all through this article.

MATERIALS AND METHODS

Bacterial strains, plasmid and culture conditions

E. coli strain JE5505 [F⁻, *lpp, pps, his, proA, argE, thi, thr, gal, lac, xyl, mtl, tsx*](4) and C600 [F⁻, *thi*-1, *thr*-1, *leu*B6, *lac*Y1, *ton*A21, *sup*E44, λ⁻](7) were used as host strains of the recombinant plasmid pIF-Ⅲ-B(1), which carries alpha-IFN gene under the control of lipoprotein promoter, *lac* promoter and operator. Schematic diagram of the plasmid pIF-Ⅲ-B is shown in Fig. 1.

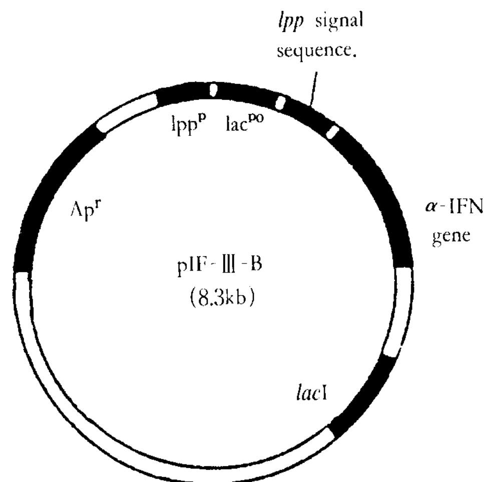

Fig. 1. Structure of plasmid pIF-Ⅲ-B.

LB medium (1% Trypton, 0.5% Yeast extract, 1% NaCl, pH 7.0) was used for all the culture of the recombinant *E. coli* strains. Ampicillin and IPTG were dissolved in distilled water at 25mg/ml and 0.1M, respectively, and sterilized with membrane filtration. The stock solution was kept at −20℃ in aliquots. They were added to culture medium simultaneously with inoculation at appropriate concentrations. In order to investigate the production of alpha-IFN and plasmid stability, the recombinante *E. coli* strains were cultivated in rotary flask shaker(New Brunswick Sci.) at 37℃. Cell growth was followed by monitoring optical density at 600 nm.

Determination of alpha-IFN activity

We followed the method described by Rubinstein, S. (5). This assay is based on the measurement of a parameter associated with cytopathic effect on host animal cells.

Preparation of samples for the assay of alpha-IFN

Three types of samples were prepared (*i. e.*, cell extract, periplasmic fraction and supernatant) to investigate the production levels of alpha-IFN and its excretion. The detailed procedures for the preparation of the samples were described previously(3).

Determination of plasmid stability

Plasmid stability tests were performed according to the procedure of Koshland *et al.* (8) with some modification. Overnight culture in LB medium supplemented with 50 ㎍/ml of ampicillin was used as an inoculum. 50 ml of fresh LB medium with or without ampicillin in 300-ml Erlenmeyer flask was inoculated with 0.1 ml of the overnight culture and shaken with 260 rpm at 37℃ in rotary shaking incubator (New Brunswick Sci.). Cell growth was monitored by measuring OD at 600 nm. After appropriate period of cultivation, 0.1 ml of the culture was transferred to 50 ml of fresh LB medium. This transfer of the culture was repeated five to nine times according to experiments. At each time of transfer of the cultures, samples were taken to measure OD and to determine plasmid stability. Appropriately diluted samples from the culture were spread on LB agar plates without and with 50 ㎍/ml of ampicillin. Stability was determined by the ratio of number of single colonies appeared on LB agar with ampicillin to number of colonies appeared on LB agar without ampicillin. In another method, single colonies formed on LB agar were transferred with sterile toothpicks to ampicillin containing LB agar and counting the number of colonies survived on the plate.

Generation number of the culture, "n", was determined by the equation of "$n=\ln(X/X_o)/\ln 2$" by measuring initial $OD(X_o)$ and final $OD(X)$.

RESULTS AND DISCUSSION

Effect of ampicillin concentration on the production of alpha-IFN

Effect of ampicillin concentration on alpha-IFN production is shown in Fig. 2. There were nearly no differences in the final cell concentration (data not shown). According to the data in Fig. 2, the recombinant cell extract prepared from the culture supplemented with 100㎍/ml of ampicillin showed the highest activity of alpha-IFN, but the activity did not differ significantly from those of extracts prepared from the rest of the cultures. The differences were nearly negligible. Supernatants and samples prepared from periplasmic fraction of the cultures also did not show significant differences according to ampicillin concentration. It was thought that the culture period was too short for the ampicillin concentration to have effects on plasmid stability and the production level of alpha-IFN. In fact, the culture period was only 4.2 generation time. Plasmids were also isolated from each culture to quantify its Plasmid content (Fig. 3), but there were no significant differences. If prolonged culture is performed, *e. g.*, fed batch culture,

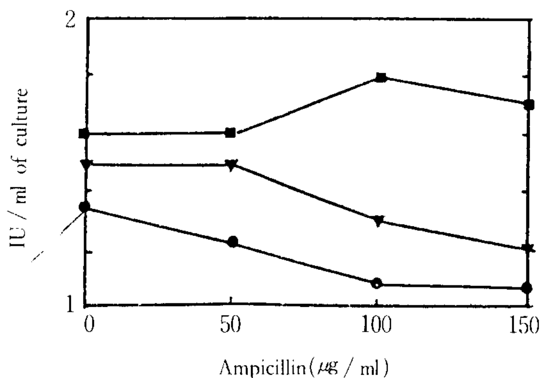

Fig. 2. Effect of ampicillin concentration of culture medium on the production of alpha-interferon.
■: Extract(10^4 IU)
●: Supernatant(10^4 IU)
▼: Periplasm(10^3 IU)

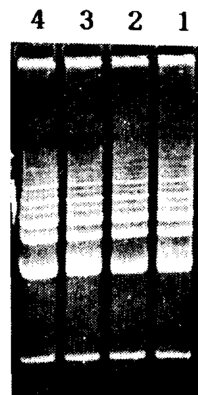

Fig. 3. Electrophoretic picture of plasmid pIF-Ⅲ-B isolated from cultures with varying concentration of ampicillin.
1. 0㎎/l, 2. 50㎎/l
3. 100㎎/l, 4. 150㎎/l

ampicillin concentration possibly has effects on the plasmid stability, and consequently alpha-IFN production level would be changed with ampicillin concentration.

Effect of IPTG concentration on the production of alpha-IFN

Effect of IPTG concentration on the alpha-IFN production is shown in Fig. 4. The total production level reached maximum at the IPTG concentration of about 0.5 mM, beyond which, no additional increase of the activity was observed. This means that 0.5 mM of IPTG is enough to counteract the effect of the repressors produced from the recombinant cells. These results correspond to those of plasmid stability test with the concentration of IPTG(data not shown). These results were obtained from 300ml skake flask cultures grown for 10 hours and the OD_{600} of the cultures reached about 3.4. When prolonged fed-batch culture is performed, adding inducer several times in appropriate quantity along with culture time course would be possibly more desirable than adding the inducer at one time, because self-degradation of inducer cannot be negligible with culture time when culture time becomes longer.

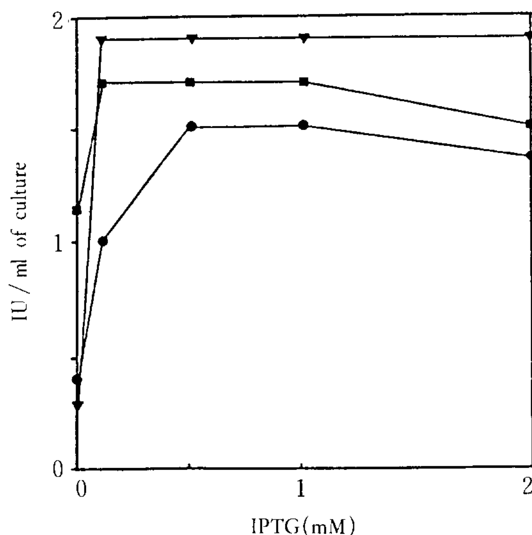

Fig. 4. Effect of inducer(IPTG) concentrations of culture medium on the production of alpha-interferon.
■: Extract(10^4 IU),
○: Supernatant(10^4. IU)
▼: Periplasm(10^3 IU)

Plasmid stability

The stability of plasmid pIF-Ⅲ-B was studied in host strains, JE5505 and C600 by following generation nember (Fig. 5). When ampicillin containing medium was used, both of the strains maintained the plasmid very stably, *i. e.*, 88% with C600 and 94% with JE5505 in 75 generations. But when medium without ampicillin was used, there was a considerable increase in segregants. The plasmid stability in JE5505 decreased to 50% in 20 generations, and then to zero % in 63 generations. The plasmid stability in C600 also decreased to 88% and 12% in 20, and 80 generations, respectively. These data show that the plasmid is relatively very stable irrespective of host strains used when they are cultured in medium with ampicillin, but there was a significant difference in the plasmid stability according to the host strains used when drug-free medium is used. Considering this fact, when recombinant plasmid is constructed for the foreign protein production, it is desirable to examine the stability of the plasmid in various host strains. According to the data, it is also desirable to use ampicillin-containing medium for the seed-culture of recombinant cell and to decrease the generation number of main culture if possible(*i. e.*, large inoculum size). It is of no use to use drug-containing medium to maintain plasmid stably when cell concentration is so high, because high concentration of the drug-degr-

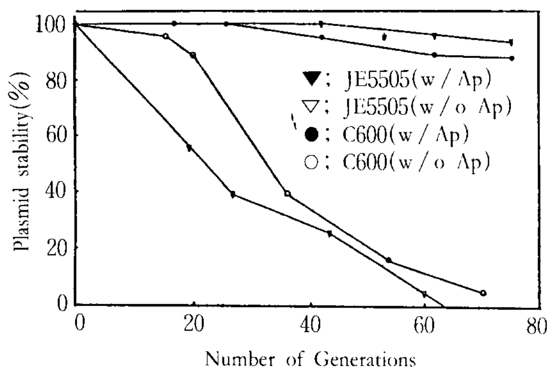

Fig. 5. Stability of plasmid pIF-Ⅲ-B in *E. coli*, JE5505 and C600 in medium with and without supplementaion of ampicillin.

ading or modifying proteins (*i. e.*, bata-lactamase in case of ampicillin) produced from the plasmid inactivate the drug contained in the medium rapidly.

Stability variation differences according to the inducer concentration added to the medium without ampicillin were also examined in JE5505 (Fig. 6). The plasmid stabilities in medium with 0.5 mM and 1.0 mM of IPTG were nearly the same in first 20 generations, even though there was some difference thereafter. This result implies that 0.5 mM of IPTG is near to saturation concentration that

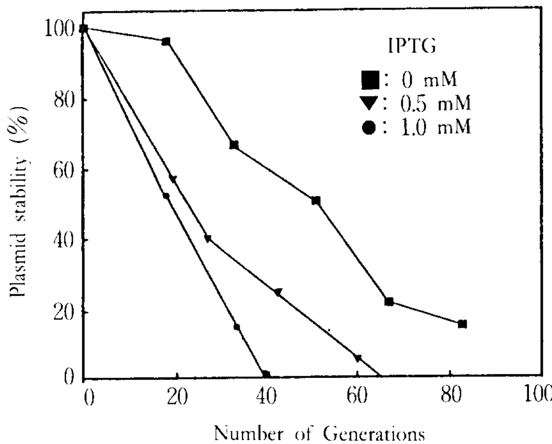

Fig. 6. Stability of plasmid pIF-Ⅲ-B in *E. coli*, JE5505 in medium with varying concentrations of IPTG.

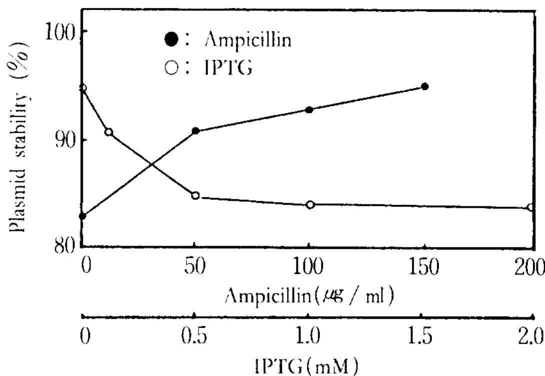

Fig. 7. Effect of ampicillin and IPTG concentration of medium on the stability of plasmid pIF-Ⅲ-B.

can has effects on the behaviors of the recombinant cell, *i. e.*, plasmid stability, cell growth and alpha-IFN production. Actually, there was no improvement on the production of alpha-IFN at concentration of IPTG above 0.5 mM as reported previously. When an inducer was not added at all, stability behavior was quite different, *i. e.*, the plasmid was maintained much more stably during the early generation number of period. This implies that the induction of alpha-IFN gene expression by adding an inducer reduces the plasmid stability significantly.

Effects of IPTG and ampicillin with broad concentration range on plasmid stability in 10 generations were shown in Fig. 7. The final OD_{600} of the cultures was ca. 3.3 irrespective of the IPTG concentration. Between 0.5 mM and 2.0 mM of IPTG they showed nearly the same plasmid stability. It was considered that 0.5 mM IPTG was near to the saturation concentration of inducer when cell concentration reached 3.3 in terms of OD. Concerning about ampicillin concentraton, there was somewhat big difference in plasmid stability between culture without ampicillin and with 50㎍/ml of ampicillin, meanwhile the stability was nearly the same at concentration above 50㎍/ml.

ABBREVIATIONS

AP^r: ampicillin resistant
IFN: interferon, IPTG: isopropyl-β-D-thiogalactopyranoside
IU: international unit, kb: kilo base pairs
lac: lactose
lpp: lipoprotein
OD: optical density

요 약

대장균의 *lpp* promoter, *lac*UV5 promoter및 operator의 조절하에 cloning된 alpha-interferon 유전자를 가지는 plasmid pIF-Ⅲ-B를 사용하여 대장균으로부터 alpha-interferon을 생산함에 있어 배지내에 ampicillin및 inducer인 IPTG의 첨가 영향을 연구하였다. Inducer인 IPTG농도 0.5 mM, ampicillin 농도 50 ㎎/*l* 에서 가장 높은 생산성을 보였다. 또한 plasmid 안정성에 있어서는 inducer인 IPTG의 경우, 0.5 mM을 경계로 안정성에 큰 차이를 보였고 ampicillin의 경우는 50 ㎎/*l* 되게 첨가했을 때는

비교적 매우 안정하였으나 첨가하지 않은 경우는 안정성이 급격히 저하되었다.

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