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Effects of Cloned Genes on the Stability of Shuttle Vectors between *Escherichia coli* and *Corynebacterium glutamicum*

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*Escherichia coli*와 *Corynebacterium glutamicum*간의 shuttle vectors의 *C. glutamicum*에서의 안정성에 대한 클론된 유전자의 영향

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ABSTRACT: ***Escherichia coli*/*Corynebacterium glutamicum* shuttle vectors, pECCG 1 and pECCG 2 were constructed by joining a 3.00 kb cryptic plasmid pCB 1 from *C. glutamicum* and a 3.94 kb plasmid pACYC 177 from *E. coli*. By trimming unessential parts and introducing mulitiple cloning site into the plasmid pECCG 1, a plasmid pECCG 122(5.1 kb) was constructed. All the shuttle vectors were stably maintained in *C. glutamicum* up to about 40 generations irrespective of kanamycin addition in the medium.**

Threonine operon(homoserine dehydrogenase/homoserine kinase) and *dapA* gene (dihydrodipicolinate synthetase) of *C. glutamicum* were cloned into the plasmid pECCG 122, and the resultant plasmids were designated pTN 31 and pDHDP 19, respectively. They were used to study the effect of cloned foreign gene on the stability of the plasmid pECCG122. Plasmids pTN 31 and pDHDP 19 were segregated rapidly from *C. glutamicum* when cultured in the medium without kanamycin. In medium with 50 μg/ml of kanamycin, their segregation rates were much slower than those in medium without kanamycin, but the kanamycin addition didn't guarantee the complete maintenance of the plasmids in *C. glutamicum*.

KEY WORDS □ **Plasmid stability, *Corynebacterium glutamicum*, Shuttle vector**

INTRODUCTION

Corynebacterium, glutamicum and the related bacteria (*Brevibacterium flavum, Brevibacterium lactofermentum*) are grampositive, so-called coryneform glutamic acid-producing bacteria and have been extensively used for the industrial production of various amino acids (Yamada *et al.*, 1972). Some coryneform bacteria are pathogens of plants and animals (Barksdale *et al.*, 1981). Despite their role in disease and their industrial importance, only limited efforts have been, until recently, dedicated to the elucidation of genetics of coryneform bacteria. Most of the genetic studies have been focused on isolating and characterizing amino acid biosynthetic mutants, including auxotrophic and analog-resistant mutants, with the objective of developing strains which produce high levels of selected amino acids.

Recent advances in biotechnology have facilitated *in vitro* construction of recombinant DNA and its introduction into a suitable host. To apply these recombinant DNA techniques to coryneform bacteria system, it is very important to develop a stable host-vector system and an efficient transformation method.

A large number of endogenous plasmids have been reported in coryneform bacteria (Martin *et al.*, 1987). However, because of their large size, multiple sites for various restriction endonucleases and their cryptic properties (*e.g.*, location of replication origin, phenotypic trait, copy number), most of them are inadequate for cloning vehicles in *C. glutamicum*. Among them, plasmids pBL 1 (Santamaria *et al.*, 1984) of *Brevibacterium lactofermentum* and pHM 1519 (Miwa *et al.*, 1984; Miwa *et al.*, 1985) of *C. glutamicum* have been widely used in the development of cloning vectors in coryneform bacteria. For the cloning vectors to be useful in industrial application, they should be stably maintained in appropriate host strains.

Although there have been a few reports on the construction of cloning vectors in coryneform bacteria, there are few on the plasmid stability in coryneform bacteria. In this paper, we describe the brief schemes for the construction of *E. coli/C. glutamicum* shuttle vectors and their stability in *C. glutamicum*. We also constructed plasmids pTN 31 and pDHDP 19 by cloning threonine operon (homoserine dehydrogenase/homoserine kinase) and *dapA* gene (dihydrodipicolinate synthetase) of *C. glutamicum*, respectively in plasmid pECCG 122 which carries multiple cloning site. The homoserine dehydrogenase and homoserine kinase are key enzymes in biosynthetic pathway of L-threonine in *C. glutamicum* (Nakamori and Shiio, 1972; Kase and Nakayama, 1972). There have been some reports on the cloning of threonine operon from coryneform bacteria to develop L-threonine-overproducing strains (Katsumata and Furuya, 1988; Morinaga *et al.*, 1987). The dihydrodipicolinate synthetase is thought to be one of the key enzymes involved in L-lysine biosynthetic pathway because it is the first enzyme for L-lysine biosynthesis from aspartate-β-semialdehyde which is a common intermediate for L-threonine and L-lysine (Cremer *et al.*, 1988). We cloned the genes in an attempt to develop amino acid-overproducing strains through gene amplification. To study the effect of cloned foreign gene on the stable maintenance of the plasmid pECCG 122 in *C. glutamicum*, we used the recombinant plasmids pTN 31 and pDHDP 19.

MATERIALS AND METHODS

Bacterial strains and plasmids

C. glutamicum JS 231 (Leu⁻, Homoserine⁻) was used as a host strain of shuttle vectors with or without cloned gene. The brief scheme for the construction of plasmids pECCG 1 and pECCG 2 is shown in Fig. 1, and pECCG 122, a derivative of pECCG1 carrying multiple cloning site in Fig. 2. Structure of plasmids pTN 31 and pDHDP 19, which were constructed by cloning threonine operon and *dapA* gene of *C. glutamicum*, respectively into plasmid pECCG 122 are shown in Fig. 3.

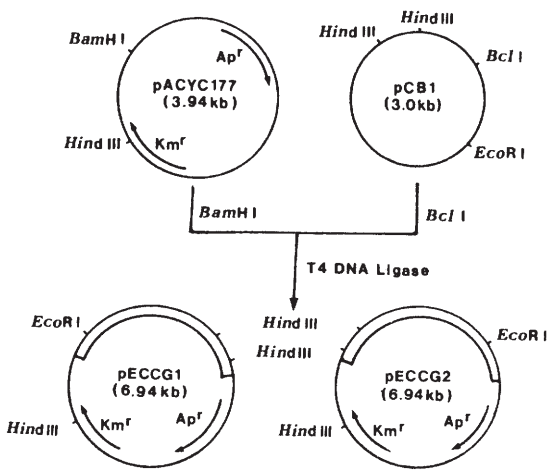

Fig. 1. *Schematic diagram for the construction of shuttle vectors pECCG 1 and pECCG 2.*

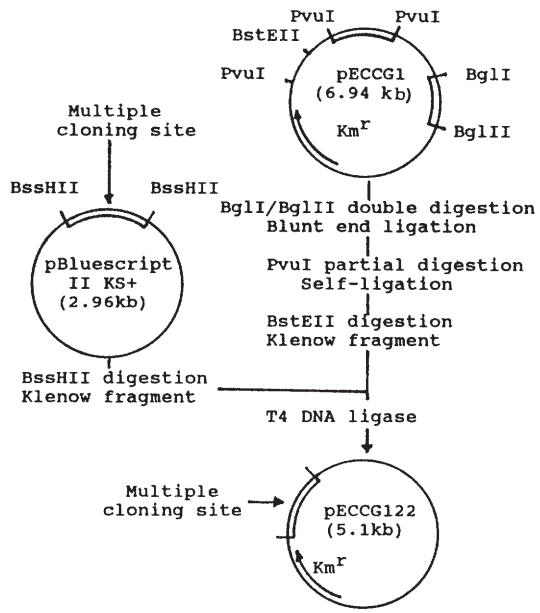

Fig. 2. *Schematic diagram for the construction of plasmid pECCG 122.*

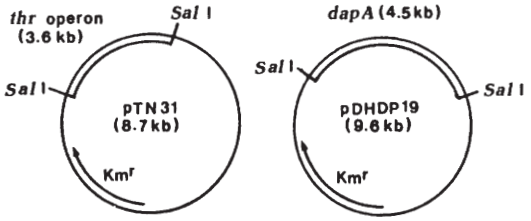

Fig. 3. *Structure of plasmids pDHDP 19*

Media and bacterial cultivation

LB medium (1% Tryptone, 0.5% Yeast extract, 1% NaCl, pH 7.0) or LB agar (1.8%) were used for the routine culture of bacteria at 30℃. To isolate plasmid DNA from *C. glutamicum*, TYG medium (1% Tryptone, 1% Yeast extract, 0.5% Glucose, 0.5% NaCl, pH 7.0) supplemented with 0.3 U/m*l* of penicillin-G was used.

Plasmid DNA preparation and transformation

Large-scale plasmid preparation from *C. glutamicum* was carried out by the alkaline lysis method of Birnboim (1983), and further purified with CsCl-ethidium bromide density gradient centrifugation (Maniatis *et al.*, 1982). Transformation of *C. glutamicum* with plasmid DNA was carried out by the electroporation method described by Noh *et al.* (1990).

Determination of plasmid stability

Overnight culture of *C. glutamicum* grown in LB medium supplemented with 50 μg/m*l* of kanamycin was used as an inoculum. Fifty m*l* of fresh LB medium with or without kanamycin in 250-m*l* Erlenmeyer flask was inoculated with 0.1 m*l* of the overnight culture and cultivated at 30℃ with shaking at 260 rpm. Cell growth was monitored by measuring an OD at 600 nm. After appropriate period of cultivation, 0.1 m*l* of the culture was transferred at 50 m*l* of fresh LB medium. This transfer of the culture was repeated four to six times. Each time of culture transfer, samples were taken and plated on LB agar with appropriate dilution.

Stability was determined by counting the number of colonies grown on LB agar and colonies grown on LB agar containing kanamycin, or transferring cells grown on LB agar to LB agar containing kanamycin and counting the number of colonies grown on the plate. Generation number of the culutre, "n", was determined by the equation of "$n=\ln(X/X_0)/\ln 2$" by measuring initial OD(X_0) and final OD(X).

RESULTS

Effect of orientation of vectors and addition of kanamycin to culture medium

As shown in Fig. 1, there are two types of possible orientation of ligation between plasmids pCB 1 and pACYC 177. The two resultant plasmids which are differ in orientation are designated as pECCG 1 and pECCG 2, respectively. To investigate the effect of kanamycin addition to culture medium on the plasmid stability, we carried out experiment with *C. glutamicum* JS 231 harboring the plasmid pECCG 1. As shown in Fig. 4, plasmid pECCG 1 was stably maintained in the strain up to about 45 generations irrespective of kanamycin addition. Also, there was no significant difference in stability between pECCG 1 and pECCG 2, indicating that orientation has

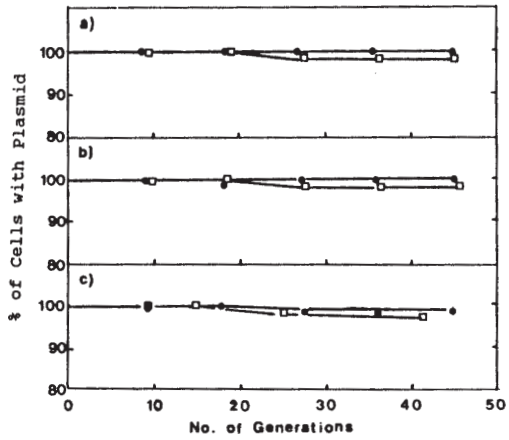

Fig. 4. *Stability of E. coli/C. glutamicum shuttle vectors.*
a) Effect of kanamycin on the stability of pECCG 1
(●—●; w/Km, □—□; w/o Km)
b) Effect of orientation on plasmid stability.
(●—●; pECCG 1, □—□; pECCG 2)
c) Plasmid stability of pECCG 1 and pECCG 122.
(●—●; pECCG 1, □—□; pECCG 122)

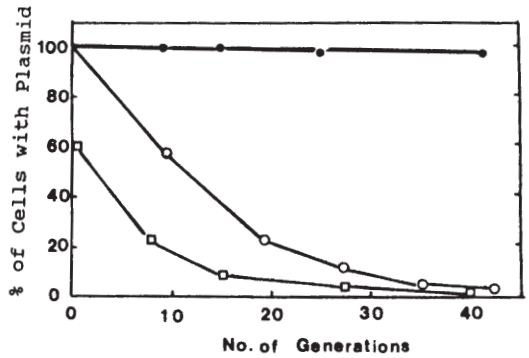

Fig. 5. *Stability of plasmids pECCG 122, pTN 31 and pDHDP 19.*
●—●; pECCG 122, ○—○; pTN 31, □—□; pDHDP 19

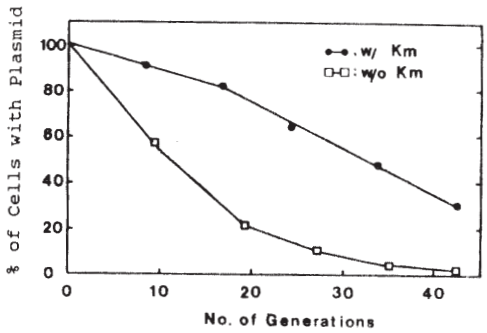

Fig. 6. *Effect of kanamycin on the stability of plasmid pTN 31.*

no effects on plasmid stability.

Fig. 2 shows the the schematic diagram for the construction of plasmid pECCG 122. Unnecessary fragments of DNA were removed from the plasmid pECCG 1 to reduce the size of the plasmid. Also, we introduced multiple cloning site useful for cloning of foreign gene into the plasmid. Plasmid pBluscript II KS+ (Stratagene, La Jolla, California, USA) was used for the DNA fragment carrying the multiple cloning site. To study the effect of this modification of pECCG 1 on the plasmid stability, *C. glutamicum* JS 231 harboring the resultant plasmid pECCG 122 was used. As shown in Fig. 4, pECCG 122 was also stable without any significant difference from pECCG 1, implying that excising parts of DNA designated in Fig. 2 and introducing multiple cloning site do not affect the stable maintenance of the resultant plasmid pECCG 122.

Effects of foreign gene in pECCG 122 on plasmid stability

To study the effect of cloning foreign gene into plasmid pECCG 122 on the plasmid stability, plasmid pTN 31 and pDHDP 19 were used. The plasmids pTN 31 and pDHDP 19 were constructed by cloning threonine operon and *dapA* gene of *C. glutamicum* into *Sal*I site of plasmid pECCG 122, respectively. Stabilities of pTN 31 and pDHDP 19 were compared as shown in Fig. 5. Both of pTN31 and pDHDP19 were unstable in *C. glutamicum*, indicating that both of threonine operon and *dapA* gene work as a serious burden for the host strain to maintain the plasmids stably. Considering that the plasmid pECCG 122 was stable, it is clear that the instability of plasmids pTN 31 and pDHDP 19 came from the cloned threonine operon and *dapA* gene. Plasmid pDHDP19 was more unstable than pTN 31, possibly because of the larger insert size or more serious imbalance in the metabolism caused by the expression of the cloned *dapA* gene in pDHDP 19. In case of pDHDP 19, even the inoculum which was grown overnight in LB

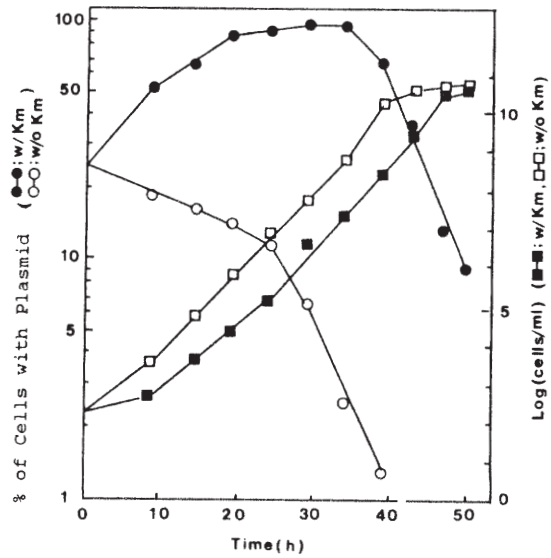

Fig. 7. *Culture time course versus stability of plasmid pDHDP 19 and cell growth in medium with or without kanamycin.*

medium containing 50 μg/ml of kanamycin, was unstable and only 60% of the cells carried the plasmid after about 10 generations.

Because plasmid pDHDP 19 was unstable even in the medium with kanamycin, experiment was carried out to see the pattern in relation with plasmid stability and cell concentration (Fig. 7). Overnight culture, of which only 25% of the cells carried plasmid, was used to inoculate fresh LB medium with or without kanamycin. Initial cell concentration was 270 cells/ml. Cells grown in the medium without kanamycin lost the plasmid rapidly and plasmid-carrying cells decreased to 1% within 40 hr. Meanwhile, cells grown in medium containing 50 μg/ml of kanamycin showed different pattern of plasmid stability. Initially, the portion of cells harboring plasmid increased up to 90% during the first 20 hr, and it was maintained at similar level for about further 15 hr and then decreased rapidly. Based on this observation, it could be concluded that during initial 20 hr of the cultivation, cells without plasmid can not grow in the medium containing kanamycin and only cells carrying plasmid can grow, and proportion of cells with plasmid increases. During the cultivation period between 20 hr and 35 hr when proportion of cells carrying the plasmid was maintained at constant, growth rates of cells with plasmid and without plasmid were thought to be similar possibly because much of the kanamycin added to the medium was inactivated by kanamycin-resistant gene products from cells harboring plasmid pDHDP 19. And it

lessened the degree of growth inhibition of cells without plasmid. As a result, plasmid stability decreased rapidly after 35 hr of cultivation. The kanamycin added to medium seems to be inactivated near to completion and cells without plasmid grow faster than cells with plasmid and begin to outnumber the cells with plasmid.

DISCUSSION

We have shown that shuttle vectors pECCG 1 and pECCG 2 which have been constructed by joining a *Bcl*I-digest of cryptic plasmid pCB 1 from *C. glutamicum* and *Bam*HI digest of plasmid pACYC 177 from *E. coli*, were very stable in *C. glutamicum* even in the absence of selection pressure. The plasmid pECCG 122 which was constructed by removing unessential parts of DNA from pECCG 1 and introducing multiple cloning site of plasmid pBluscript II KS+, was also stably maintained in *C. glutamicum*. These results imply that a series of works in relation with the construction of the vectors did not impair the normal function of replication origin of plasmid pCB 1, and the introduction of plasmid pACYC 177 did not impose any serious burden for the host cells as to segregate the plasmids.

Cloning of foreign genes, *i.e.*, threonine operon and *dapA* gene of *C. glutamicum* in plasmid pECCG 122 decreased the plasmid stability dramatically indicating that the cloned genes were serious burden for the host strain to maintain the plasmid. Actually, growth rate of cells carrying plasmid pTN 31 or pDHDP 19 was much slower than that of cells without plasmid. Even when cells with plasmid were grown in medium with kanamycin, the cells lost the plasmids in some generations of period. This indicates that addition of kanamycin to culture medium, which was thought to be a strong tool for stable maintenance of plasmids, is no longer effective toward the end of culture. However it reduced the segreation rate of plasmids. It was speculated that the above has resulted from the inactivation of kanamycin by the kanamycin resistant gene products (aminoglycoside phosphotrasferase) from cells harboring the plasmids.

적 요

C. glutamicum 유래의 cryptic plasmid pCB 1과 *E. coli* 유래의 plasmid pACYC 177을 사용하여 2가지 orientation의 shuttle vector pECCG 1과 pECCG 2를 제작하였다. pECCG 1으로부터 불필요한 부분을 제거하고 cloning에 유용한 multiple cloning site를 도입함으로써 5.1 kb 크기의 plasmid pECCG 122를 제작하였다. 제작된 모든 vector들은 kanamycin이 첨가되지않은 배지에서 배양했을때 *C. glutamicum* 내에서 *40 generation* 기간 동안 매우 안정하게 유지되었다.

Plasmid pECCG122에 *C. glutamicum*의 threonine operon및 *dapA* gene을 cloning하여 재조합 plasmid pTN 31및 pDHDP 19를 각각 제작하였다. 재조합 plasmid pTN 31및 pDHDP 19는 kanamycin이 첨가되지않은 배지에서 배양했을때 *C. glutamicum*내에서 매우 불안정했으며 kanamycin을 50 μg/ml 되게 첨가한 배지에서는 plasmid의 소실 속도가 다소 지연되기는 했으나 장시간 안정하게 유지되지는 않았다.

REFERENCES

1. **Barksdale, L.,** 1981. The Prokaryotes (Starr, M. P. *et al.* eds), pp. 1827-1837, Springer-Velag, New York.
2. **Cremer, J., C. Treptow, L. Eggeling and H. Sahm,** 1988. Regulation of enzyme of lysine biosynthesis in *C. glutamicum*. *J. Gen. Microbiol.*, **134,** 3221-3229.
3. **Birnboim, H.C.,** 1983. A rapid alkaline extraction method for the isolation of plasmid DNA, pp. 234-255, Methods in Enzymology, Vol. 100, Academic Press, New York.
4. **Kase, H. and K. Nakayama,** 1972. Production of L-threonine by analog-resistant mutants. *Agric. Biol. Chem.*,**36,** 1611-1621.
5. **Katsumata, R. and A. Furuya,** 1988. Genetic engineering in amino acid-producing corynebacteria. *8th International Biotechnology Symposium*, vol. **1,** 147-158.
6. **Maniatis, T., E.F. Fritsch and J. Sanbrook,** 1982. Molecular cloning: a Laboratory Manual. pp. 93, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
7. **Martin, J.F., R. Santamaria, H. Sandoval, G. del, Real, L.M. Mateos, J.A. Gil and A. Aguilar,** 1987. Cloning systems in amino acids-producing corynebacteria. *Bio/Technology*, **5,** 137-147.
8. **Miwa, K., H. Matsui, M. Terabe, S. Nakamori, K. Sano and H. Momose,** 1984. Cryptic plasmid in glutamic acid-producing bacteria. *Agric. Biol. Chem.*, **48,** 2237-2903.
9. **Miwa, K., K. Matsui, M. Terabe, K. Ito, M. Ishida, H. Takagi, S. Nakamori and K. Sano,** 1985. Construction of novel shuttle vectors and a cosmid vector for the glutamic acid-producing bacteria *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*. *Gene*, **39,** 281-286.
10. **Morinaga, Y., H. Takagi, M. Ishida, K. Miwa, T. Sato, S. Nakamori, and K. Sano,** 1987. Threonine

producton by co-existence of cloned genes coding homoserine dehydrogenase and homoserine kinase in *Brevibacterium lactofermentum*. *Agric. Biol. Chem.*, **51**, 93-100.

11. **Nakamori, S. and I. Shiio,** 1972. Microbial production of L-threonine. *Agric. biol. Chem.*, **36**, 1209-1216.
12. Noh, K.S., S.J. Kim, H.H. Lee, H.H. Hyun and J.H. Lee, 1990. High frequency electroporation-transformation system of coryneform bacteria. *Korean J. Biotechnol. Bioeng.* **5**, 299-306.
13. Santamaria, R., J.A. Gil, J.M. Mesas and J.F. Martin, 1984. Characterization of endogenous plasmid and development of cloning vectors and a transformation system in *Brevibacterium lactofermentum*, *J. Gen. Microbiol.* **130**, 2237-2246.
14. Yamada, K., S. Kinoshita, T. Tsunoda and K. Aida, 1972. The Microbial Production of Amino Acids. Halsted Press, New York.

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