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Construction of a *Corynebacterium glutamicum-Escherichia coli* Shuttle Vector and Cloning the Homoserine Dehydrogenase Gene from *C. glutamicum*

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Corynebacterium glutamicum-Escherichia coli Shuttle Vector 개발과 *C. glutamicum*의 Homoserine Dehydrogenase Gene Cloning

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Abstract — A 7.5 kilobases hybrid plasmid, designated as pCE1301, was constructed by combining *Escherichia coli* plasmid pBEL1 which carries the kanamycin resistance gene of Tn5 with a cryptic plasmid, pSR1 of *Corynebacterium glutamicum*. pCE1301 was transformed *C. glutamicum* by PEG-mediated protoplast method and its transformation efficiency was about 3.0×10^3 transformants per μg of the hybrid plasmid DNA. The physical map reveals that pCE1301 has single restriction sites for *Sal*I and *Eco*RI, respectively. The kanamycin resistance of pCE1301 was stably maintained in *C. glutamicum* up to 25 generations and any segregation was not detected. pCE1301 was also introduced into *Brevibacterium flavum* and *E. coli*, and replicated in those strains. pCE1301 was proved to be useful in cloning the homoserine dehydrogenase gene from *C. glutamicum*.

Coryneform bacteria such as *C. glutamicum*, *B. flavum* and *B. lactofermentum* are widely known as industrial producers of various amino acids. Newly overproducing strains of these bacteria were prepared chiefly by random mutagenesis and screening auxotrophic and/or regulatory mutanats. Recently, this classical strain improvement method was complemented by the application of recombinant DNA technology to this group of organism. For this purpose, host-vector system had to be established. The discovery of the small multicopy plasmids in *B. lactofermentum* (1, 2) and *C. glutamicum* (1, 3) made construction of the cloning vectors for coryneform bacteria and development of the transformation system (2-6). Marker genes used for the construction of the vectors replicating in coryneform bacteria usually originated from *E. coli*, *Bacillus subtilis* and *Streptomyces*. Some of them conferring kanamycin, chloramphenicol and hygromycin resistance (2, 5, 7) are expressed well in coryneform bacteria, while others are expressed weakly (3, 8, 9). Many of the vectors constructed are structurally unstable (8, 10) and/or rapidly lost in the host cells during cultivation under non-selective conditions (8, 9).

In this paper we report on the construction and properties of a stable broad-host-range vector carrying stably an efficiently expressed gene conferring kanamycin resistance in coryneform bacteria and on the cloning the homoserine dehydrogenase gene from *C. glutamicum*.

Key words: Shuttle vector, *C. glutamicum-E. coli*, homoserine dehydrogenase gene cloning
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Materials and Methods

Plasmids and bacterial strains

pBEL1 (11) carrying Km resistance of Tn5 and pSR1 (9) were isolated from *E. coli* KF3901 and *C. glutamicum* ATCC13058, respectively. *E. coli* HB 101, *C. glutamicum* ATCC13059 and *B. flavum* ATCC15168 were used as the hosts for this shuttle vector.

Growth media and enzymes

E. coli was grown at 37℃, while *C. glutamicum* and *B. flavum* were grown at 30℃ in LB medium (12). Minimal medium of *C. glutamicum* for cloning the gene encoding homoserine dehydrogenase was glucose 5g, ammonium sulfate 1.5g, magnesium sulfate 1g, biotin 15 mg, thiamine 10 mg, ferrous sulfate 10 mg, manganese sulfate 1 mg, zinc sulfate 0.5 mg and agar 15g in 1000 m*l* distilled water. For the selection of transformants, kanamycin of 25 μg/m*l* was added to the SB regeneration medium (9). Restriction endonucleases, T4-DNA ligase and calf intestinal phosphatase were purchased from Promega (U.S.A.) and KOSCO (Korea), and used as described in the instructions of the manufactures.

DNA isolation and manipulation

Plasmid DNA from *E. coli* was prepared according to the method of Birnboim and Doly (13). A modified protocol described by Katsumata *et al.* (4) except substituting by LB medium containing 2% glucose and 2% glycine was used for the large-scale preparation of plasmid DNA from *C. glutamicum* and *B. flavum*. Plasmid for the transformation was purified by ethidium bromide/cesium chloride density gradient centrifugation. For rapid screening of plasmid DNA in *C. glutamicum*, the alkaline lysis procedure from the method of Birnboim and Doly was modified by combined incubation of 5 mg/m*l* of lysozyme and 1 mg/m*l* of archromopeptidase (Wako Pure Chemical Ltd.) for 30 min. Chromosomal DNA from *C. glutamicum* was prepared according to the method of Follettie and Sinskey (14). Gene clean kit (BIO 101 Inc., U.S.A) was used for DNA purification from agarose gel. Electrophoresis of DNA was carried out in agarose gel according to the method of Maniatis *et al.* (12).

Transformation

E. coli cells were transformed with the method of Maniatis *et al.* Transformation of coryneform strains was done as previously described (9) except substituting by prolonged lysozyme treatment of 12 hours.

Assay of homoserine dehydrogenase activity

Homoserine dehydrogenase activity was determined according to the method of Miyajima and Shiio (15).

Southern hybridization

The hybridization was performed after the procedure of described previously (12) with [α-^{32}P]dCTP and a kit provided by Amersham International Plc for random hexanucleotide primed synthesis.

Determination of plasmid stability

C. glutamicum ATCC13059 (pCE1301) and *C. glutamicum* ATCC13059 (pCT2146) were cultured in 100 m*l* LB broth, and the kanamycin resistance and structural segregation was investigated every five generations.

Estimation of plasmid copy number

The copy number of plasmid per *C. glutamicum* chromosome was calculated using densitometric evaluation of the photographed agarose gel electrophorograms of the total DNA according to the method of Projan and Carlton (16). 2,800 kb for *C. glutamicum* (14) was the value of chromosome size used in the calculations.

Results

Construction of a shuttle vector

For the construction of a shuttle vector for *E. coli* and coryneform bacteria, plasmid pSR1 of *C. glutamicum* ATCC13058 was chosen as a coryneform replicon. A 3.0 kb cryptic plasmid, pSR1 has single sites for *Bcl*I, *Bgl*II and *Eco*RI. pBEL1, a 4.5 kb deletion plasmid having pACYC184 replication

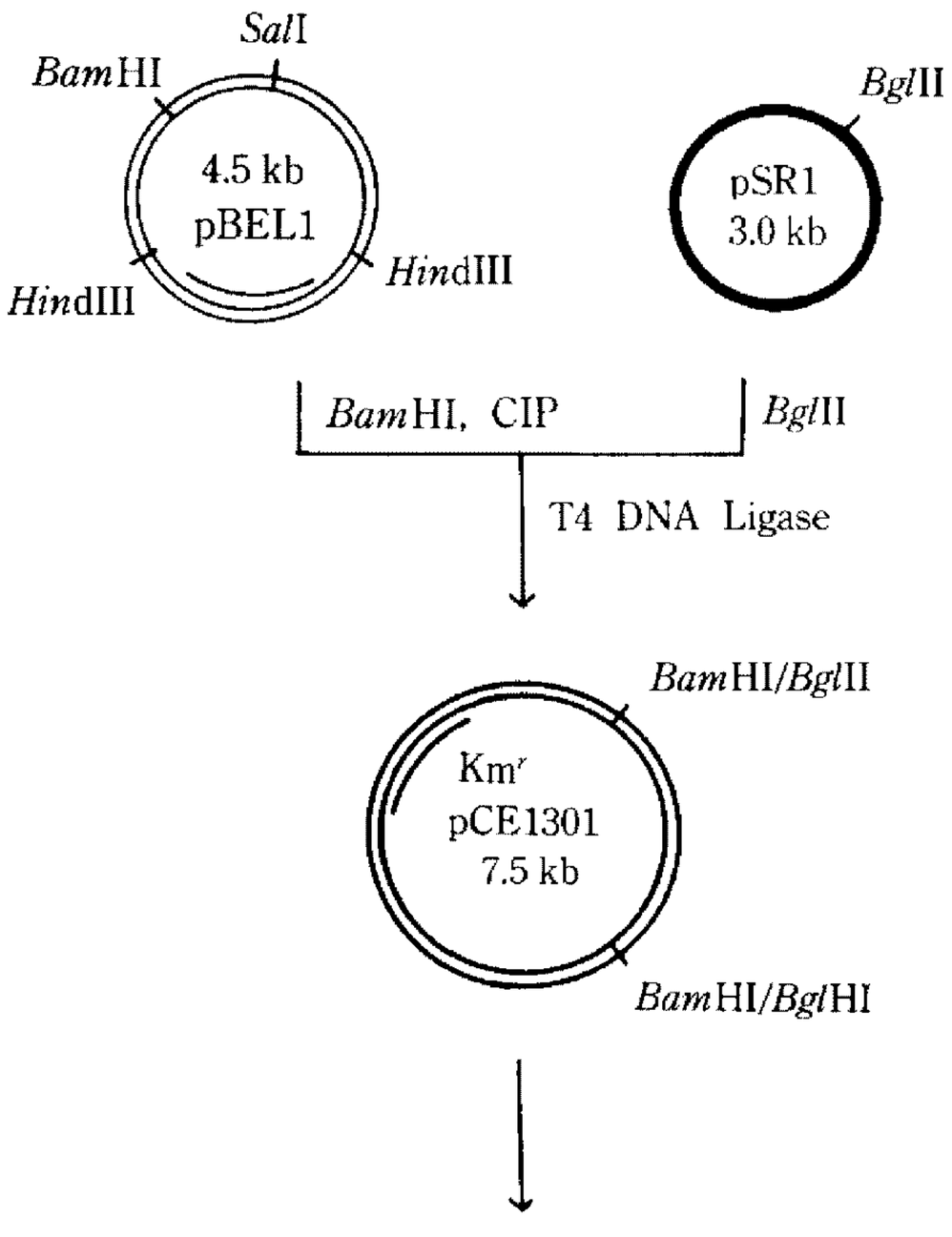

Fig. 1. Construction of hybrid plasmid pCE1301.
*Bgl*II-digested pSR1 was cloned at the *Bam*HI site of pBEL1 in *E. coli* HB101. The pCE1301 of 7.5 kb carries kanamycin resistance gene, and CIP means calf intestinal phosphatase.

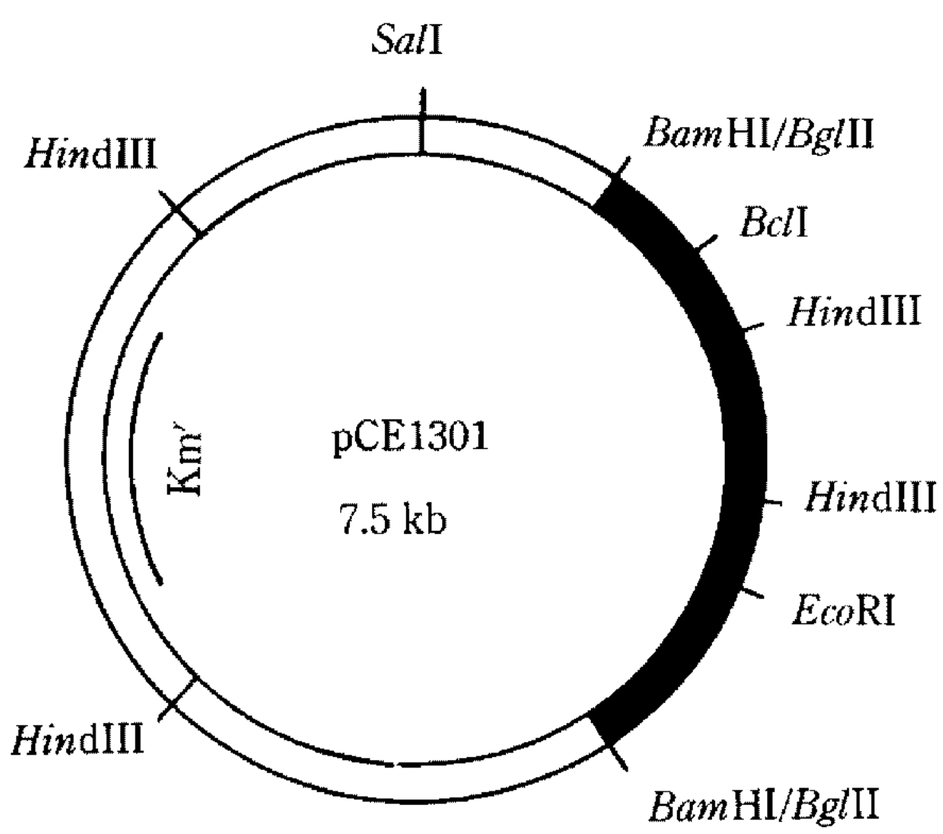

Fig. 2. Restriction map of recombinant plasmid pCE 1301.
Closed line indicates pSR1 digested by *Bgl*II and open line represents *Bam*HI-digested pBEL1 carrying kanamycin resistance gene.

origin and kanamycin resistance gene, has single restriction endonuclease sites of *Bam*HI and *Sal*I,

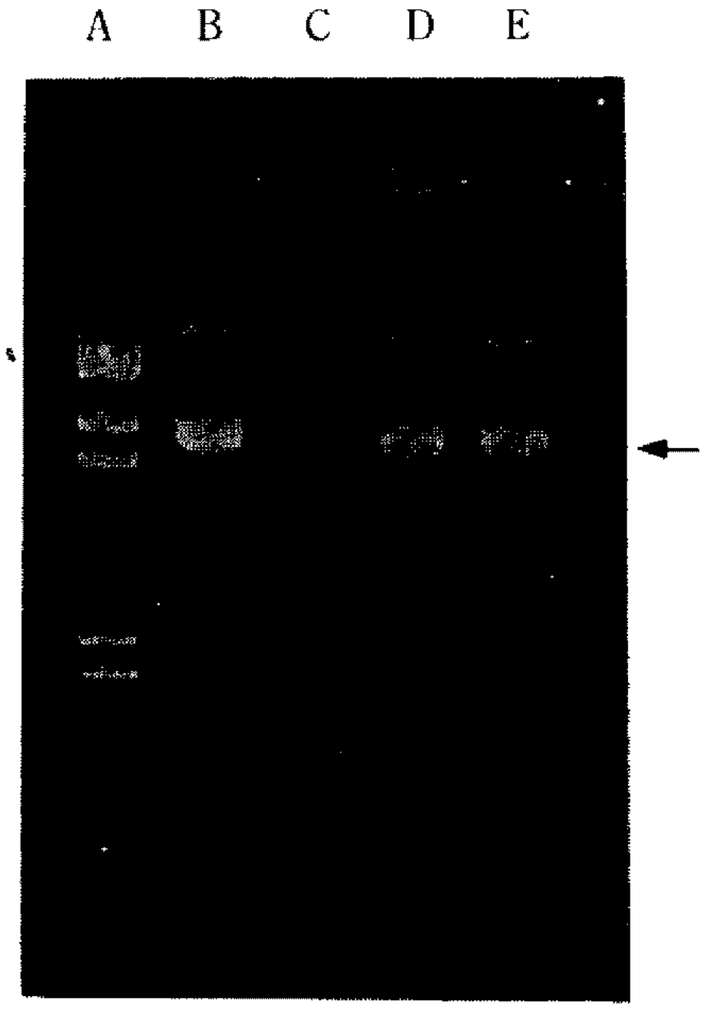

Fig. 3. Identification of pCE1301 from coryneform bacteria.
pCE1301 isolated from *E. coli* Km^r transformant was introduced into plasmid-free *C. glutamicum* ATCC 13059 and *B. flavum* ATCC15168, and again prepared from the transformants as shown on the agarose gel (1%, w/v). Arrow indicates pCE1301 prepared from each transformant by alkaline extraction method. Lane A:λ-*Hin*dIII DNA, size marker. Lane B: pCE1301 from *E. coli* HB101 (pCE1301). Lane C: no plasmid from *C. glutamicum* ATCC13059. Lane D: pCE1301 from *C. glutamicum* ATCC13059 (pCE1301). Lane E: pCE1301 from *B. flavum* ATCC15168 (pCE1301).

respectively. pSR1 was linearized by digestion with *Bgl*II, *Bam*HI isoschizomer and ligated into the 4.5 kb *Bam*HI fragment of the pBEL1 and then transformed into *E. coli* HB101 (Fig. 1). Recombinant plasmids containing pBEL1 were identified by analyzing the plasmids from Km^r transformants. Three of the 20 transformants with that phenotype contained the 7.5 kb plasmid in size, whose size corresponded to the summation of pSR1 and pBEL1. One of them was designated as pCE1301. The physical map of pCE1301 was established by the restriction endonuclease analysis (Fig. 2).

Transformation of pCE1301 into coryneform bacteria

pCE1301 isolated from the *E. coli* transformant was used for transformation of coryneform bacteria (Fig. 3). Plasmid pCE1301 was transferred into pla-

smid-free *C. glutamicum* ATCC13059 by PEG-mediated protoplast transformation. However, the efficiency of transformation with pCE1301 isolated from the *E. coli* transformant was extremely low (10~20 transformants/μg of plasmid DNA). When pCE1301 prepared from the *C. glutamicum* transformant was used for *C. glutamicum* transformation again, the efficiency of this homospecific transformation was relatively high (about 3,000 transformants/μg of plasmid DNA), which may indicate the presence of the restriction system in *C. glutamicum* ATCC13059. Vector pCE1301 was further transformed plasmid-free *B. flavum* ATCC15168 and replicated.

Determination of pCE1301 copy number

The copy number of plasmid pCE1301 in *C. glutamicum* ATCC13059 was determined according to the method of Projan and Carlton. The number was about 45 per chromosome and smaller than that of parental plasmid pSR1 of 140 copies (1).

Cloning of the homoserine dehydrogenase gene from *C. glutamicum*

Follettie *et al.* (17) reported that the homoserine dehydrogenase gene (*thr*AB) from *C. glutamicum* AS019 existed in a 3.6 kb *Sal*I chromosomal fragment. To prove the utility of pCE1301 as a useful cloning vector, we attempted to clone the *thr*AB gene from *C. glutamicum* according to this information. *Sal*I fragments, ranging from two to ten kb of *C. glutamicum* ATCC13059 chromosomal DNA (3 μg), were purified from agarose gel with Gene Clean Kit and Ligated with the *Sal*I-cleaved and dephosphorylated pCE1301 (1 μg). The resulting ligation mixture was used for transformation of *C. glutamicum* AS172 (kindly provided by A.J. Sinskey of MIT). Of about 1,000 transformants, we selected a positive clone which complemented a mutant of *C. glutamicum* AS172 lacking homoserine dehydrogenase activity. The plasmid isolated from the transformant, designated as pCT2146, contained the identical 3.6 kb *Sal*I fragment carrying the homoserine dehydrogenase gene. Presence of the *thr*AB gene on plasmid pCT2146 was confirmed by retransformation, restriction endonuclease analysis (Fig. 4), and Southern hybridization (Fig. 5). This result represents that pCE1301 can be used for cloning genes of useful enzymes in coryneform bacteria.

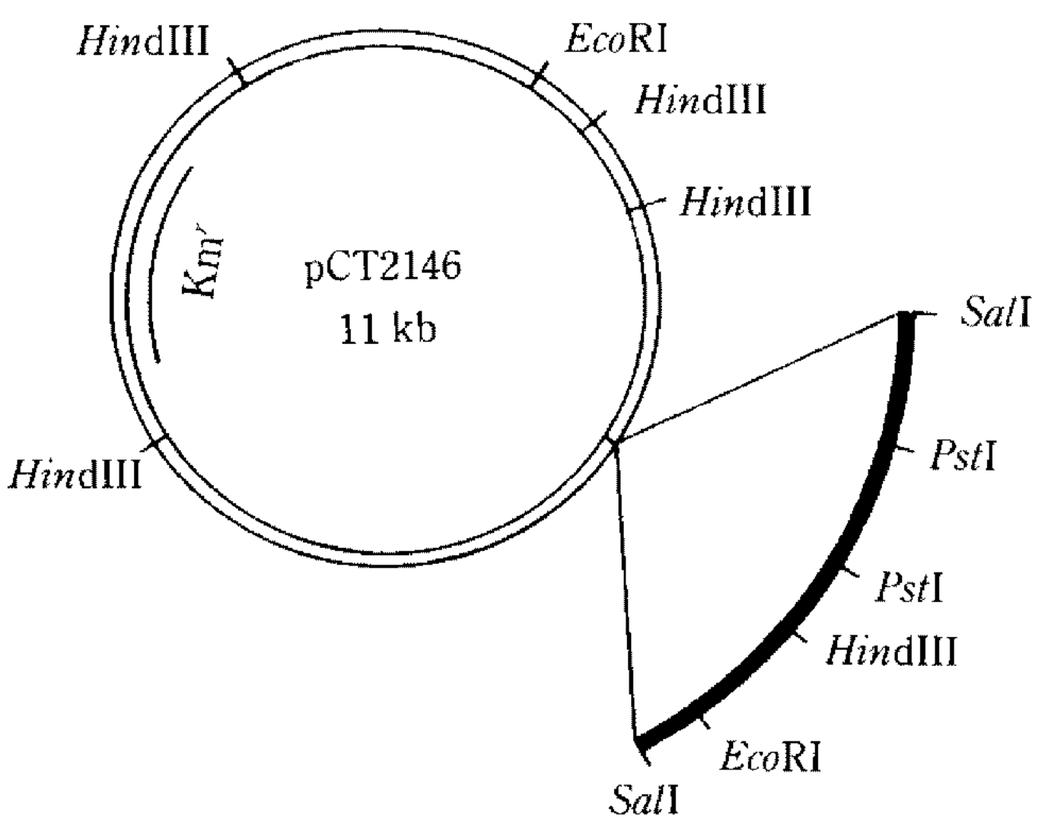

Fig. 4. Restriction map of pCT2146 harboring *thr*AB of *C. glutamicum* ATCC13059.

Overproduction of homoserine dehydrogenase

The homoserine dehydrogenase activity in crude extracts of *C. glutamicum* ATCC13059 was compa-

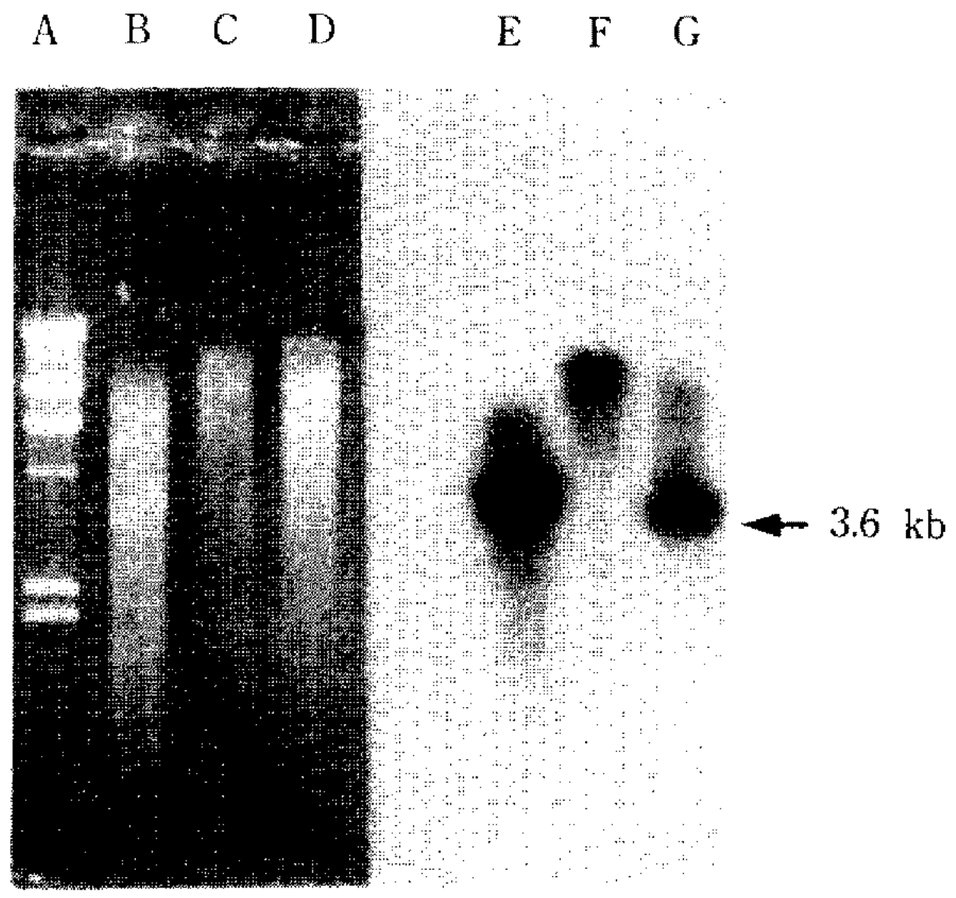

Fig. 5. Southern hybridization analysis of *C. glutamicum thr*AB gene.

Chromosomal DNA of *C. glutamicum* ATCC13059 was digested by *Eco*RI (lane B), *Hind*III (lane C) and *Sal*I (lane D) and then hybridized with the probe from 3.6 kb *Sal*I fragment of pCT2146 (lane E, F, G), respectively. Hybridization spot at 3.6 kb position between *Sal*I-digested *C. glutamicum* ATCC13059 chromosomal DNA and the probe is shown at lane G (arrow). Lane A is λ-*Hin*dIII size marker.

Table 1. Homoserine dehydrogenase activity of crude extracts

	Cells of *C. glutamicum* ATCC13059		
	Without	pCE1301	pCT2146
Homoserine dehydrogenase activity	4.2	4.5	90.5*

*Homoserine dehydrogenase activity was expressed as μmoles of oxidized NADPH/min/mg protein.

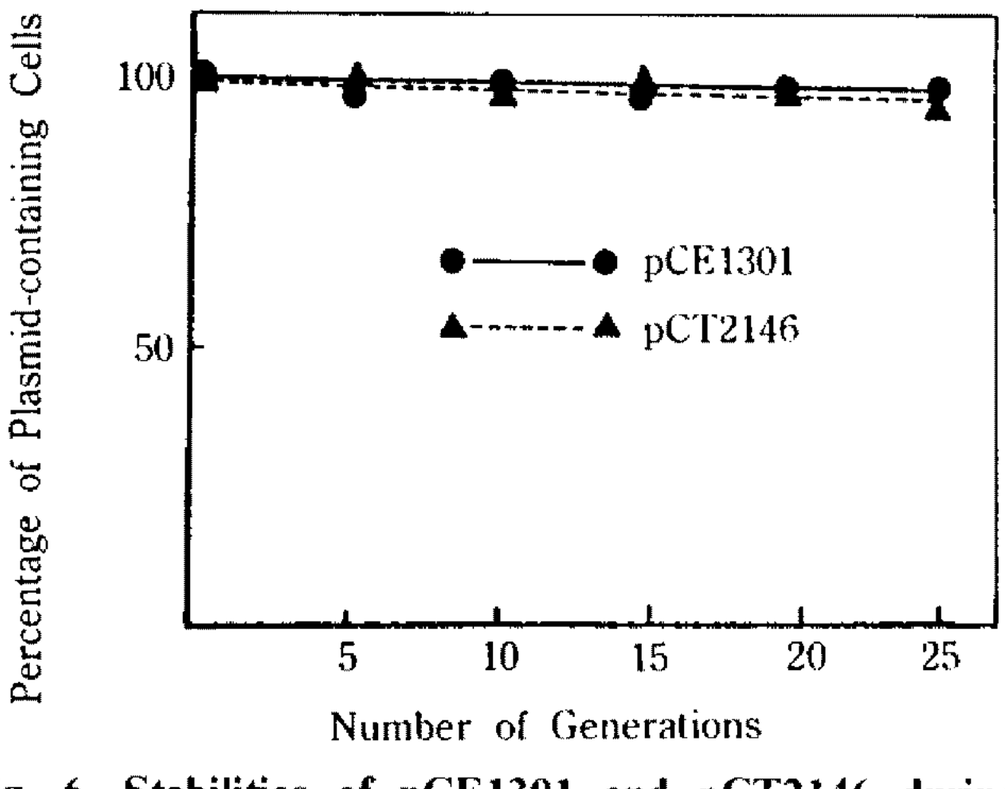

Fig. 6. Stabilities of pCE1301 and pCT2146 during cultivation.
C. glutamicum ATCC13059 (pCE1301) and *C. glutamicum* ATCC 13059 (pCT2146) were subcultured consecutively in LB broth without kanamycin every five generations up to 25 generations. The transformants were transferred on the LB agar plates without kanamycin and toothpicked on the plates with kanamycin. The persentage represents the ratio of kanamycin resistant versus total cell number.

red with that of *C. glutamicum* ATCC13059 (pCT 2146). The activity was increased up to 22 fold at *C. glutamicum* ATCC13059 (pCT2146) (Table 1).

Plasmid stability

To determine plasmid stabilities of pCE1301 and pCT2146, we checked the segregational stability with kanamycin resistance and structural stability analyzed with the electrophoretic analysis. Under the complete medium without the addition of kanamycin, pCE1301 and pCT2146 showed a remarkedably high plasmid stability as shown Fig. 6 (nearly over 96%) and any structural unstability was not detected during 25 generations (data not shown).

Discussion

There have been many reports about the construction of cloning vectors in coryneform bacteria. However, any detailed studies have not almost been carried out about the plasmid stability, especially that of recombinant plasmids which are generally unstable and easily lost during fermentation. Antibiotics are good at increasing the plasmid stability, but are so expensive that their use is not practical for large-scale fermentation. Amino-acid production of long-term batch fermentation condition is done through four or five consecutive steps. Therefore, we subcultured consecutively five times the transformants carrying pCE1301 and pCT2146 without the selective pressure of kanamycin, and did not find out any plasmid unstability. This high efficiency of the plasmid maintenance system from the observed stabilities of pCE1301 and pCT2146 may come from the cryptic plasmid pSR1 of *C. glutamicum*, judging from the reports of Patek (18) and Japan patent 63279791 from Asahi-chemical. They suggested that the stabilities of the hybrid plasmids during 20 or 30 generations would come from the cryptic plasmids from *B. flavum* and *C. melassecola*. However, it was not revealed until now what a factor of pCE1301 engenders such a high stability over other vectors. The other important characteristic of pCE1301 is that it is a *B. flavum-C. glutamicum-E. coli* shuttle vector. In the future, the utility of pCE1301 as a cloning vector will be more increased after improvement in the lack of restriction endonuclease sites and the second genetic marker on the vector and in the low transformation efficiency. Passenger DNA may be cloned into unique *Sal*I restriction site. Multiclong sites for the restriction endonucleases, if to be incorporated into the *Sal*I of pCE1301, will faciliate the construction of the genomic banks. As the second genetic marker, erythromycin would be one of the candidates which was expressed in *B. lactofermentum, C. glutamicum* and *E. coli* (19). The transformation efficiency of pCE1301 with the protoplast transformation system, 3×10^3 transformants of μg plasmid DNA, is relatively low in comparison with that of 10^4 to 10^6 transformants of μg plasmid DNA in other reports. The

efficiency is mainly dependent upon the protoplast-formation level because of their rigid cell wall and regeneration. That will be increased if the improved method such as the electrophoratic transformation is adopted. Despite relatively low transformation efficiency of pCE1301, the cloning vector has allowed the isolation of specific amino acid biosynthetic gene encoding homoserine dehydrogenase from *C. glutamicum*. The vector appears to be a stable vehicle for gene transfer and maintenance of foreign genes in coryneform bacteria.

요 약

Tn5의 kanamycin 저항성 유전자를 가진 pBEL1 plasmid와 *C. glutamicum* cryptic plasmid인 pSR1으로 7.5 kb의 새로운 plasmid를 만든 후, 이를 pCE1301이라 명명하였다. 이 pCE1301은 PEG-protoplast법으로 *C. glutamicum*을 형질전환하였을 때 효율이 약 3×10^3 형질전환체/μg이었으며 *Sal*I과 *Eco*RI 제한효소 절단부위가 1개씩이었다. 또 Km이 없는 배지에서 25세대까지 안정하게 유지되었으며 *B. flavum, E. coli*에서 복제되었다. 이 pCE1301을 이용하여 *C. glutamicum*의 homoserine dehydrogenase 유전자를 cloning하였다.

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References

1. Miwa, K., H. Maitui, M. Terabe, S. Nakamori, K. Sano and H. Momose: *Agric. Biol. Chem.* **48**, 2901 (1984).
2. Santamaria, R.I., J.A. Gil, J.M. Mesas and J.F. Martin: *J. Gen. Microbiol.* **130**, 2237 (1984).
3. Ozaki, A., R. Katsumata, T. Oka and A. Furuya: *Mol. Gen. Genet.* **196**, 175 (1984).
4. Katsumata, R., A. Ozaki, T. Oka and A. Furuya: *J. Bactriol.* **159**, 306 (1984).
5. Miwa, K., H. Matsui, M. Terabe, K. Ito, M. Shida, H. Takagi, S. Nakamori and K. Sano: *Gene*, **39**, 281 (1985).
6. Santamaria, R.I., J.A. Gil and J.F. Martin: *J. Bacteriol.* **162**, 463 (1985).
7. Santamaria, R.I., J.F. Martin and J.A. Gil: *Gene*, **56**, 199 (1987).
8. Smith, M.D., J.L. Flickinger, D.W. Lineberger and B. Schmidt: *Appl. Environ. Microbiol.* **51**, 634 (1986).
9. Yoshihama, M., K. Higashiro, E.A. Rao, M. Akedo, W.G. Shanabruch, M.T. Follittie, G.C. Walker and A.J. Sinskey: *J. Bacteriol.* **162**, 591 (1985).
10. Batt, C.A., W.S. Shanabruch and A.J. Sinskey: *Biotechnol. Lett.* **7**, 717 (1985).
11. Yoo, I.D. *et al.*: KAIST report BS N7042-91-3, 80-116 (1988).
12. Maniatis, T., E. Fritsch and J. Sambrook: *Molecular Cloning: A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982).
13. Birnboim, H.C. and J. Doly: *Nucleic Acids Res.* **7**, 1513 (1979).
14. Follettie, M.T. and A.J. Sinskey: *J. Bacteriol.* **167**, 695 (1986).
15. Miyajima, R. and I. Shiio: *J. Biochem.* **68**, 311 (1970).
16. Projan, S.J., S. Carleton and R.P. Novick: *Plasmid.* **9**, 182 (1983).
17. Follettie, M.T., H.K. Shin and A.T. Sinskey: *Mol. Microbiol.* **2**, 53 (1988).
18. Patek, M., O. Navratil, J. Hochmannova, J. Nesvera and J. Hubacek: *Biotechnol. Lett.* **11**, 231 (1989).
19. Yeh, P., J. Oreglia, F. Prevots and A.M. Sicard: *Gene*, **47**, 301 (1986).

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