Construction of a Chimeric Plasmid in *Bacillus subtilis*

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Staphylococcal plasmids pTP4 (2.7 megadaltons encoding resistance to chloramphenicol) and pTP5 (2.6 megadaltons encoding resistance to tetracycline), which replicate and express resistance in *B. subtilis*, were found to cut by *Hind*III endonuclease respectively at a single site and three sites. A chimeric plasmid pTA1245 (4.1 megadaltons) was constructed from pTP4 and pTP5 by *Hind*III digestion and ligation with *E. coli* DNA ligase. pTA1245 expresses resistances to chloramphenicol and tetracycline in *B. subtilis*, and pTA1245 is amplified in the presence of tetracycline. A physical map of pTA1245 was constructed.

Recently, certain antibiotic resistant plasmids originally detected in *Staphylococcus aureus* have been transformed into *B. subtilis*.^{1~5)} These plasmids could be usable as molecular cloning vectors in *B. subtilis*. We have also tried to construct a molecular cloning system in *B. subtilis*, and have already obtained a useful host strain *B. subtilis* RM125⁶⁾ which lost its restriction and showed high efficiency in transformation with heterologous DNAs of *Bacillus* species.⁷⁾ This paper reports construction of a chimeric plasmid presumably usable as a cloning vector in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. subtilis* RM125 (*arg*₁₅⁻, *leu*A₈⁻, *hsr*M, *hsm*M) was used as a recipient strain in this experiment. *B. subtilis* LMAH761222 (*leu*⁻, *met*B⁻, *ade*⁻, *his*A⁻) strains carrying pTP4 and pTP5, respectively, were kindly supplied by Dr. Megumi Kono (Tokyo College of Pharmacy).

Media. NY medium used for bacterial growth was described previously.⁶⁾ Media for preparation of competent cells of *B. subtilis* were prepared as described.⁷⁾ Antibiotic concentration used for selection were 10 µg/ml for both chloramphenicol (Cm) and tetracycline (Tc).

Isolation and characterization of plasmid DNA. Plasmid DNA from *B. subtilis* was isolated by the method of Tanaka *et al.*⁸⁾ Characterization of plasmid DNA and

plasmid DNA fragments were performed by electrophoresis on vertical 0.8% agarose (Wako Pure Chemical) gels. Restriction endonucleases *Hind*III, *Eco*RI and *Bam*I were purchased from Boehringer Mannheim. *Bsu*G was prepared as previously described.⁹⁾ *Sal*I was prepared by the method of Arrand *et al.*¹⁰⁾

Transformation of plasmid DNA. Frozen competent cells of *B. subtilis* RM125 were prepared as described.¹¹⁾ DNA uptake and incubation for expression were performed as described previously.⁵⁾

Ligation procedures. One µg of pTP4 DNA and 3 µg of pTP5 DNA were separately digested with *Hind*III for 90 min at 37°C. pTP4 DNA was completely digested and pTP5 DNA was partially digested in this condition. Digested DNAs were mixed and precipitated by two volumes of cold ethanol and dissolved in 100 µl of ligation buffer.¹²⁾ *E. coli* DNA ligase (20 µl) which was prepared by the method of Modrich *et al.*¹³⁾ was added to the mixture. After the mixture was incubated at 11°C for 48 hr, ligated DNA was precipitated by cold ethanol and dissolved in TES (50 mM Tris, 10 mM NaCl, 5 mM EDTA, pH 7.5). This solution was mixed with competent cells of *B. subtilis* RM125 for transformation.

Biohazard procedures. This experiment was carried out under P1 containment level as specified by the Ministry of Education, Science and Culture of Japan.

RESULTS

Isolation and analysis of chimeric plasmid

Plasmids pTP4 (1 µg) and pTP5 (3 µg) were separately digested with *Hind*III, mixed and treated with *E. coli* DNA ligase. After in-

FIG. 1. Construction of pTA1245.

Plasmids pTP4 and pTP5 were digested with *Hind*III, followed by ligation with *E. coli* DNA ligase. The ligation mixture was used to transform *B. subtilis* RM125. A plasmid extracted from a Tc^rCm^r transformant was named as pTA1245. The structure of pTA1245 was determined by following experiment (Figs. 2 and 3). Symbols (—) *Eco*RI site; (▶) *Hind*III site.

FIG. 2. Cleavage Patterns of pTA1245.

Plasmid DNAs were digested with restriction endonucleases. Digested fragments were analyzed by agarose gel electrophoresis (0.8% agarose gel, 80V, 2 hr). 1 and 5, *Hind*III-digested lambda DNA fragments as the standards of molecular weights; 2, pTP4 DNA digested with *Eco*RI; 3, pTA1245 DNA digested with *Eco*RI; 4, pTA1245 DNA digested with *Hind*III; 6, pTP5 DNA digested with *Hind*III; 7, pTP4 DNA digested with *Eco*RI and *Hind*III; 8, *Eco*RI-digested lambda DNA.

cubation at 11°C for 48 hr, some ligation products were observed by agarose gel electrophoresis (data not shown). The mixture was used to transform *B. subtilis* RM125. In this experiment, only one Cm^r and Tc^r transformant was obtained. The plasmid pTA1245 DNA isolated from this

transformant was digested with *Hind*III and *Eco*RI restriction endonucleases. As shown in Fig. 1 and Fig. 2, pTA1245 was digested with *Eco*RI at only one site, and 4.1 megadaltons fragment was observed. *Hind*III digestion of pTA1245 produced A and C fragments of pTP5 and 2.4 megadaltons fragment. This 2.4 megadaltons fragment seemed to be a deleted fragment of pTP4, and this was designated as pTP4'. This deleted plasmid pTP4' was often obtained after transformation of *B. subtilis* RM125 with pTP4 native DNA.

Plasmids pTP4, pTP5 and pTA1245 DNAs were not digested by *Bam*I, *Bsu*G and *Sal*I restriction endonucleases.

In order to investigate the orientation of pTP4', pTP5-A and pTP5-C fragments in pTA1245, pTA1245 was linearized with *Eco*RI and digested further with *Hind*III. *Hind*III digestion was performed at 22°C, and partial digestion products were analyzed by agarose gel electrophoresis. Because a fragment of 0.5 megadaltons was observed at 20~45 min of digestion, the orientation of pTA1245 was determined as Fig. 3.

Characterization of pTA1245

Covalently closed circular pTA1245 DNA was used to transform *B. subtilis* RM125. Cm^r, Tc^r transformants were obtained at an efficiency of about 10⁴ transformants/μg DNA. This efficiency was almost the same as that of transformation with pTP4 or pTP5 plasmid DNA (10³~10⁴ transformants/μg DNA).

It was reported that tetracycline resistant plasmid pTP5 was amplified when Tc was added to the growth medium, and the ratio of the plasmid DNA to chromosomal DNA reached 38~68%.¹⁴⁾ In order to investigate the effect of Tc on the copy number of pTA1245, plasmid DNA prepared from *B. subtilis* RM125 (pTA1245) grown under four conditions (no drugs, presence of Cm, presence of Tc, and presence of Cm and Tc) were examined by agarose gel electrophoresis. As shown in Fig. 4, the copy number of pTA1245 was increased by Tc treatment. This fact shows that pTA1245 is replicated under control of

FIG. 3. Partial Digestion of pTA1245.

Plasmid pTA1245 DNA was linearized by *Eco*RI digestion. This linear DNA was digested further with *Hind*III at 22°C.

Symbols: (—) *Eco*RI site; (▴) *Hind*III site.

FIG. 4. Effects of Drugs on Copy Number of pTA1245.

B. subtilis RM125 was grown in 10 ml of NY medium under four conditions (1, no drugs; 2, presence of 10 µg/ml of Cm; 3, presence of 10 µg/ml of Tc; 4, presence of Cm (10 µg/ml) and Tc (10 µg/ml)). Cleared lysates were prepared from these cultures and examined by agarose gel electrophoresis. The copy number of pTA1245 increased when Tc was added to the growth medium.

pTP5 replicator in the presence of Tc.

DISCUSSION

In this study, a chimeric plasmid pTA1245 constructed from pTP4 and pTP5 by “*Hind*III-ligase method” was found to replicate and inherit stably in *B. subtilis*. This result indicates that pTP4 and/or pTP5 can be vector(s) of molecular cloning in *B. subtilis*. Especially, it was found that the replicator of pTP5 is active in the chimeric plasmid pTA1245, and it is highly amplified in the presence of tetracycline. The plasmids pTP5 and pTA1245 will be useful vectors for cloning of foreign DNA in *B. subtilis*, because of the amplifiability.

Molecular cloning vectors are required to have high transforming efficiencies. The transforming efficiencies of pTA1245 and pTP5 are

about 10^4 transformants/µg DNA. This ratio is not sufficient, because cloning procedures consist of many steps those decrease transforming efficiencies of plasmids. So it is necessary to increase the transforming efficiencies of vector plasmids.

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