Cloning and Expression of a Gene for Resistance to Butirosin both in *Escherichia coli* and *Bacillus subtilis*

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We have cloned a butirosin resistance gene located on a 2.9 Md *Hind*III fragment of the chromosomal DNA of *Bacillus vitellinus* Z-1159, a butirosin producer, on a shuttle vector, pTA1302 (5.2 Md, Ap^r, Cm^r). The recombinant plasmid conferred resistances to butirosin, neomycin and kanamycin, on both *Escherichia coli* and *Bacillus subtilis*. Functional expression of aminoglycoside-3'-phosphotransferase activity from the butirosin resistance gene was confirmed in the cell extracts of both *E. coli* and *B. subtilis* harboring the recombinant plasmid. A cleavage map of the recombinant plasmid was constructed.

Recently, molecular cloning experiments and construction of host-vector systems of *Bacillus subtilis* have been widely performed.^{1~4)} We also constructed a host-vector system of *B. subtilis*⁵⁾ and some shuttle vectors useful for molecular cloning experiments both on *Escherichia coli* and *B. subtilis*. In this paper, we report the cloning of a DNA fragment encoding resistance to butirosin and other aminoglycoside antibiotics, derived from the chromosome of butirosin-producing microorganism *B. vitellinus*, using a shuttle vector, pTA1302, as a cloning vehicle. The utility of our shuttle vector, pTA1302, was confirmed by the present work.

MATERIALS AND METHODS

Bacterial strains and plasmids. Butirosin-producing microorganism *B. vitellinus* Z-1159 was kindly supplied by Takeda Chemical Industries, Ltd. (Osaka, Japan). *E. coli* C600 r⁻1 m⁻ (*leu*⁻, *thr*⁻, *thi*⁻) and *B. subtilis* RM125 (*hsrM*, *hsmM*, *arg15*, *leu*)⁶⁾ were used as recipient strains for cloning experiments. The shuttle vector, pTA1302, used in this experiment was constructed in our laboratory. This plasmid is a recombinant plasmid of pBR322 and

pTP4.⁷⁾ The scheme of construction and a physical map of this plasmid are shown in Fig. 1.

Media and transformation procedures. All bacteria were grown in NY medium.⁶⁾ Competent cells of *E. coli* C600 r⁻1 m⁻ were prepared by the method of Horinouchi *et al.*⁸⁾ After transformation, cultures were incubated at 37°C for 90 min, and then plated onto NY agar containing Cm (10 µg/ml), Ap (50 µg/ml) and Bt (10 µg/ml). Transformation of *B. subtilis* protoplasts was performed according to the method of Chang and Cohen.⁹⁾ After expression for 90 min, protoplasts were plated on DM3 regeneration medium containing Cm (20 µg/ml) and Bt (50 µg/ml).

Isolation of DNAs. Chromosomal DNA of *B. vitellinus* Z-1159 was isolated by the method of Saito and Miura.¹⁰⁾ Plasmid DNAs were isolated from *B. subtilis* as previously described.¹¹⁾ Plasmid DNAs in *E. coli* were prepared according to Horinouchi *et al.*⁸⁾ Large scale preparation of plasmid DNAs was performed by CsCl-ethidium bromide density gradient centrifugation.

Restriction endonuclease digestion and ligation. Restriction endonucleases *Eco*RI and *Hind*III were purchased from Boehringer Mannheim. Digested DNAs were analyzed by agarose gel electrophoresis (0.7% agarose [Seakem]). T4 DNA ligase was purchased from Boehringer. Ligation procedures were performed accord-

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Abbreviations: Cm, Tc and Bt: chloramphenicol, tetracycline and butirosin, respectively. Resistances to these antibiotics are expressed as Cm^r, Tc^r and Bt^r, respectively. Md: megadaltons.

FIG. 1. Schematic Presentation of Construction of Shuttle Vectors.

In this figure, we describe five recombinant plasmids. Recombinant plasmids between pTP4 and pBR322 were constructed by the *Hind*III and/or *Eco*RI—ligase methods in *E. coli*. These plasmids were all useful in *E. coli* as vectors. But in *B. subtilis*, although plasmids pTA1302, pTA1311, pTA1321 and pTA1322 were stable, pTA1312 was not stably maintained. In this case, some deleted plasmids were recovered from the host (unpublished data). This instability did not depend on the *recE4* gene in the host cell. (*B. subtilis* RM141 (a *recE4* derivative of *B. subtilis* RM125) was used as recipient strain in this experiment.)

In this study, we used pTA1302 as a cloning vehicle, because this plasmid has single *Eco*RI and *Hind*III sites, respectively. Underlined antibiotic resistance in this figure indicates that this resistance is not expressed in *B. subtilis*.

ing to the supplier's recommendations.

Hybridization procedures. Three micrograms of chromosomal DNA of *B. vitellinus* Z-1159 was digested with *Eco*RI and *Hind*III respectively and electrophoresed on a 0.7% agarose slab gel in TBE buffer (50 mM Tris-borate (pH 8.3), 1 mM EDTA). Blotting of DNA fragments from alkali-denatured agarose gel onto a nitrocellulose membrane (S & S Co.) was carried out with an electroblotting system (Wakamori Co.) in TAE buffer (40 mM Tris-acetate (pH 7.8), 20 mM sodium acetate, 1 mM EDTA) at 0.2 to 0.5 A for 2 to 3 hr. DNA fragments were fixed to the membrane in a vacuum oven at 80°C for 2 hr.

Labelling of the recombinant plasmids was performed with a nick translation kit (Amersham) using 32 P-dCTP (Amersham) according to the supplier's recommendations. Hybridization was performed as follows. DNA-fixed nitrocellulose filters were preincubated in 5 ml of modified Denhardt's solution (5×SSC containing 0.1% (w/v) of bovine serum albumin, ficoll, polyvinylpyrrolidone and SDS, and 50 µg/ml of sonicated calf thymus DNA) at 62°C for 16 to 18 hr. 3 ml of 32 P-labeled plasmid DNA solution in modified Denhardt's solution (approximately 5×10^6 dpm of probe per 2×11 cm filter) was

added to the preincubation mixture and incubation was continued for an additional 16 to 18 hr at 62°C. After washing with 2×SSC containing 0.1% SDS, filters were dried at room temperature. Exposure to Kodak XAR-5 X-ray film was done with an intensifying screen (Dupont, Lightning Plus) at room temperature.

Assay of aminoglycoside-3'-phosphotransferase activity. Preparation of cell free extracts from *B. vitellinus*, *E. coli* and *B. subtilis*, and assaying of aminoglycoside-3'-phosphotransferase activities were performed as previously described.¹²⁾ The antibiotic butirosin was kindly supplied by Takeda Chemical Industries, Ltd. (Osaka). Kanamycin B and tobramycin (3'-deoxy-kanamycin B) were gifts from Meiji Seika, Ltd. (Kawasaki, Japan).

RESULTS

Cloning of DNA fragments encoding resistance to butirosin in E. coli

Three micrograms of *B. vitellinus* chromosomal DNA was partially digested with *Hind*III and mixed with 1 µg of *Hind*III-digested pTA1302. T4 DNA ligase (1 unit) was

added to the mixture (100 μ l) which was then incubated at 22°C for 1 hr. The ligated DNA was transformed into 0.5 ml of competent cells of *E. coli* C600 r^-m^- . After expression in NY medium at 37°C for 90 min, cultures were plated onto NY agar containing Ap (50 μ g/ml), Cm (10 μ g/ml) and Bt (10 μ g/ml), and 11 transformants were isolated. Plasmid DNAs were prepared from these transformants, and their molecular sizes and cleavage patterns were investigated with *Eco*RI and *Hind*III. The eleven recombinant plasmids were classified into 4 types, pTABR4, pTABR5, pTABR6 and pTABR10 by the sizes of insertion fragments. Cleavage patterns of these plasmids are shown in Fig. 2 and cleavage maps are summarized in Fig. 3. All the four recombinant plasmids contained, on the vector plasmid pTA1302, a *Hind*III fragment (4.6 Md) in common, which was derived from

FIG. 2. Cleavage Patterns of pTABR4, pTABR5, pTABR6 and pTABR10.

1 and 8, lambda DNA fragments digested with *Hind*III; 2, *Eco*RI-digested pTA1302 and *Eco*RI plus *Hind*III digested pTA1302 were mixed. Other columns were *Eco*RI plus *Hind*III digested DNAs. 3 and 6, pTABR4; 4, pTABR5; 5, pTABR6; 7, pTABR10. Agarose gel electrophoresis was carried out on 0.7% agarose slab gel.

FIG. 3. Cleavage Maps of pTABR4, pTABR5, pTABR6 and pTABR10 (size in megadaltons).

FIG. 4. Schematic Presentation of Construction of pTABR17.

pTABR4 was digested with *Eco*RI and *Hind*III to generate six fragments. Vector pTA1302 was digested with *Eco*RI, and mixed with fragments of *Eco*RI plus *Hind*III digested fragments of pTABR4. Only two fragments (2.9 Md and 1.2 Md) would be easily cloned into the *Eco*RI site of pTA1302, because they have two *Eco*RI cohesive ends. After ligation, the reaction mixture was transformed into *E. coli* C600 $r^- m^-$. Ap^rCm^rBt^r transformants were selected and plasmid DNAs were recovered from them. In this experiment, we obtained only one type of recombinant plasmid which had the 2.9 Md fragment derived from *B. vitellinus* in the *Eco*RI site of pTA1302. This plasmid was designated as pTABR17. No Bt^r transformants which had the 1.2 Md fragment were obtained.

B. vitellinus (this is confirmed in the next section). pTABR4 and pTABR10 were composed of only the 4.6 Md *Hind*III fragment and vector pTA1302. But their orientations were reversed. pTABR5 and pTABR6 contained, in addition to the 4.6 Md fragment, some other *Hind*III fragments which seemed to have no functions in the butirosin resistance. The butirosin-resistant gene was deduced to be coded on the 4.6 Md fragment, because all the recombinant plasmids contained this fragment. The molecular size of 4.6 Md seemed to be larger than that needed to code a single gene, so we tried to obtain a

smaller fragment by subcloning. As shown in Fig. 3, 4.6 Md fragment can be cut by *Eco*RI into 4 fragments (2.9, 1.2, 0.3 and 0.2 Md). To clone 2 large fragments (2.9 and 1.2 Md) into the *Eco*RI cutting site of pTA1302, pTABR4 was cut by *Hind*III and *Eco*RI and mixed with *Eco*RI-digested pTA1302, followed by ligation treatment. Competent cells of *E. coli* C600 $r^- m^-$ were transformed with the ligation mixture, and Ap^rCm^rBt^r transformants were selected. As shown in Fig. 4 (schematic presentation), only the 2.9 Md or 1.2 Md fragment would be easily ligated into the *Eco*RI site of pTA1302, because they have two *Eco*RI ends.

FIG. 5. Cleavage Pattern of pTABR17.

1 and 8, lambda DNA fragments digested with *Hind*III; 2, pTABR4 digested with *Hind*III; 3, pTABR4 digested with *Eco*RI; 4, pTABR17 digested with *Hind*III; 5, pTABR17 digested with *Eco*RI; 6, pTABR4 digested with *Eco*RI and *Hind*III; 7, pTA1302 digested with *Eco*RI. Agarose gel electrophoresis was carried out on 0.7% agarose slab gel.

In this experiment, we obtained Ap^rCm^rBt^r transformants carrying the 2.9 Md fragment but not the 1.2 Md fragment on pTA1302 (Fig. 5). This means that the *Eco*RI fragment of 2.9 Md codes the gene for resistance to butirosin. The hybrid plasmid containing the 2.9 Md fragment was designated as pTABR17 (Fig. 4).

Transforming abilities of 5 recombinant plasmids for *E. coli* C600 r⁻m⁻ were tested. As shown in Table I, the 5 plasmids showed almost the same transforming efficiencies for *E. coli*. Ratios of Ap^rCm^rBt^r transformants to Ap^rCm^r (character of pTA1302) transformants varied among plasmids, but they were all almost one. It was found that these plasmids were able to replicate and express their resistances in *E. coli*. In fact, plasmid DNAs prepared from these transformants showed the same molecular sizes and the same restriction patterns as the original plasmids used for transformation (data not shown).

B. vitellinus Z-1159, a butirosin producing strain, was known to be resistant not only to butirosin but also to other related aminoglycoside antibiotics,¹²⁾ because of the substrate specificity of its aminoglycoside-modifying enzyme. So we tested plasmid-carrying *E. coli* as

TABLE I. TRANSFORMING EFFICIENCIES OF pTABR PLASMIDS IN *E. coli* COMPETENT CELLS

One microgram of pTABR plasmids was added to 0.5 ml of competent cells (1×10^8 cells/ml) of *E. coli* C600. DNA uptake was performed at 0°C for 30 min and at 37°C for 10 min. 1 ml of NY medium was added to the competent cell mixture which was then incubated at 37°C for 90 min. Transformants were scored on NY plates containing Ap (50 µg/ml) and Cm (10 µg/ml), and NY plates containing Ap, Cm and Bt (10 µg/ml), respectively.

Plasmid	Transformants/ml		Ratio (b/a)
	Ap ^r Cm ^r (a)	Ap ^r Cm ^r Bt ^r (b)	
pTABR4	1.6×10^5	1.3×10^5	0.81
pTABR5	5.4×10^4	2.0×10^4	0.37
pTABR6	4.6×10^4	5.0×10^4	1.09
pTABR10	5.7×10^4	3.3×10^4	0.58
pTABR17	1.2×10^5	8.3×10^4	0.69

to the resistance to neomycin (Nm) and kanamycin (Km). *E. coli* C600 r⁻m⁻ and *E. coli* C600 r⁻m⁻ carrying pTA1302, a vector plasmid, were sensitive to Bt, Km and Nm. *E. coli* C600 r⁻m⁻ carrying pTABR4, pTABR5, pTABR6, pTABR10 and pTABR17, respectively, were all resistant to these 3 antibiotics. It was confirmed that the aminoglycoside-modifying enzyme derived from *B. vitellinus* gene showed the same activity in *E. coli*. This fact is confirmed enzymatically in the last section of this paper.

Southern hybridization analysis of cloned DNAs

In order to confirm that the cloned fragments were really derived from *B. vitellinus* Z-1159, hybridization analysis was performed among recombinant plasmids and chromosomal DNA of *B. vitellinus*. Plasmid DNAs of pTA1302, pTABR4, pTABR5, pTABR6 and pTABR17 were labeled by nick translation and hybridized to *Hind*III or *Eco*RI digested chromosomal DNA of *B. vitellinus*. (pTABR10 was not used as a probe because this plasmid was composed of pTA1302 and the same 4.6 Md fragment as pTABR4.) As shown in Fig. 6, all the plasmids except pTA1302 hybridized to the 4.6 Md fragment of *Hind*III digestion and the 2.9 Md fragment of *Eco*RI digestion of the *B. vitellinus* chromo-

FIG. 6. Southern Blot Hybridization Analysis among pTABR Plasmids and Chromosomal DNA of *B. vitellinus* Z-1159.

EcoRI or *HindIII* digested chromosomal DNA was loaded on a horizontal 0.7% agarose slab gel. Electrophoresis was performed at 2 V/cm for 16 hours in TBE buffer. After alkali denaturation, blotting of DNA fragments onto a nitrocellulose membrane and hybridization procedures were performed as described in MATERIALS AND METHODS. 1, lambda-*HindIII* fragments; 2, *EcoRI* digested chromosomal DNA of *B. vitellinus*; 3, *HindIII* digested chromosomal DNA of *B. vitellinus*. A to E were autoradiographs of Southern blots with ^{32}P -labeled pTA1302 (A), pTABR4 (B), pTABR5 (C), pTABR6 (D) and pTABR17 (E).

some in common. pTABR4, pTABR5 and pTABR6 also hybridized to the 1.2 Md fragment of *EcoRI* digestion in common. This fragment corresponds to the 1.2 Md *EcoRI* fragment involved in the 4.6 Md *HindIII* fragment (see Fig. 3). The 2.3 Md band in lane D3 corresponds to the 2.3 Md *HindIII* fragment in pTABR6. The 2.2 Md *EcoRI* band in lane C2 has no direct corresponding band in pTABR5, because a possible corresponding fragment in pTABR5 (the upper $0.3+0.2+0.5$ Md fragment or the lower $0.2+0.7$ Md fragment in Fig. 3) has already been cut by *HindIII* and does not have the original *EcoRI* cutting feature. In case of pTABR17, ^{32}P -labeled pTABR17 only hybridized to 2.9 Md and 4.6 Md fragments because this recombinant plasmid was composed of the vector and 2.9 Md *EcoRI* fragment. The weak positive hybridization bands corresponding to molecular weights larger than 15 Md in lanes B3,

C3, D3 and E3 might be produced by non-specific hybridization, because there is much DNA mass at this position as can be seen in lane 3 of the agarose gel. From these results, it was confirmed that pTABR plasmids really contained chromosomal DNA of *B. vitellinus* Z-1159.

Transformation of pTABR plasmids into B. subtilis protoplasts

To test expression of the butirosin resistance gene in *B. subtilis*, five pTABR plasmids were transformed into protoplasts and competent cells of *B. subtilis* RM125. When competent cells were used as recipients, Cm^r transformants were obtained at low efficiencies, but Cm^rBt^r transformants were not obtained (Ap^r of pTA1302 is not expressed in *B. subtilis*). When protoplasts were used as recipients, transformants were obtained as shown in Table II. The number of transformants select-

TABLE II. TRANSFORMING EFFICIENCIES OF pTABR PLASMIDS IN *B. subtilis* PROTOPLASTS

Protoplasts of *B. subtilis* RM125 were prepared according to the method of Chang and Cohen.⁹⁾ One microgram of pTABR plasmids was mixed with 0.5 ml of a protoplast suspension (approximately 10^9 protoplasts/ml) and polyethyleneglycol-mediated DNA uptake was performed. Transformants were scored on DM3 regeneration agar plates containing Cm (20 μ g/ml) or both Cm (20 μ g/ml) and Bt (50 μ g/ml). The regeneration efficiency of protoplasts was about 1% in this experiment.

Plasmid	Transformants/ml	
	Cm ^r	Cm ^r Bt ^r
pTABR4	1030	646
pTABR5	910	490
pTABR6	270	176
pTABR10	3000	1100
pTABR17	3×10^4	1×10^4
pTA1302	1.8×10^5	0

ed by Cm^rBt^r was almost the same as the number of transformants selected by Cm^r for each plasmid. Cleared lysates were prepared from these transformants and their plasmid DNAs were analyzed by agarose gel electrophoresis. Almost all the plasmids prepared from transformants with 5 pTABR plasmids were somewhat smaller than the original plasmids (data not shown). Only one Cm^rBt^r transformant which had the same size of plasmid DNA as the donor plasmid DNA was obtained on transformation with pTABR10. In order to confirm the stability of the pTABR plasmids in *B. subtilis*, plasmid DNAs prepared from Cm^rBt^r transformants of *B. subtilis* were transferred again into *E. coli* C600 r⁻m⁻. Plasmid DNAs recovered from the *B. subtilis* transformants with pTABR5, pTABR6, pTABR10 and pTABR17 DNAs were able to produce Ap^rCm^rBt^r transformants of *E. coli*.

FIG. 7. Assay of Aminoglycoside 3'-Phosphotransferase Activity (I).

(A): Cell-free extracts were prepared from 100 ml of late log phase cultures (approximately 5×10^8 cells/ml) of *B. vitellinus* Z-1159, *B. subtilis* RM125, *B. subtilis* RM125 (pTABR10), *E. coli* C600 and *E. coli* C600 (pTABR10). Reactions were performed in 500 μ l of 100 mM phosphate buffer (pH 7.5) containing 20 mM ATP, 40 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 4 mM butirosin and 250 μ l of cell free extract at 37°C for 60 min. Reactions were stopped by heating at 80°C for 10 min. After centrifugation supernatants were analyzed for residual butirosin activities on paper discs. The indicator strain used in this experiment was *B. subtilis* ATCC 6633. (B): Inactivation was also examined by thin-layer chromatography. 1, control (no cell-free extract was added); cell free extract of 2, *B. vitellinus*; 3, RM125; 4, RM25 (pTABR10); 5, C600; 6, C600 (pTABR10) was added, respectively.

But in the case of pTABR4, plasmid DNAs recovered from *B. subtilis* could not transform *E. coli* to Ap^rCm^rBt^r nor Ap^rCm^r. Some fragment of the pBR322 portion in the hybrid plasmid pTABR4 which is essential for replication in *E. coli* seems to be lost during replication in *B. subtilis*.

All the pTABR plasmids recovered from *B. subtilis* except pTABR4 retained replicating abilities in *E. coli* as well as their characteristic resistance genes. However, a small deletion often seemed to have occurred during the replication in *B. subtilis*. Only pTABR10 retained its original structural integrity without any alteration in size or restriction pattern in *B. subtilis*.

Assay of aminoglycoside-3'-phosphotransferase activity

In order to confirm the expression of the Bt^r

gene in both *E. coli* and *B. subtilis*, aminoglycoside-3'-phosphotransferase activities in cell free extracts of *B. subtilis* RM125, *B. subtilis* RM125 (pTABR10), *E. coli* C600, *E. coli* C600 (pTABR10) and butirosin-producing strain *B. vitellinus* Z-1159 were examined. After incubation of butirosin in a reaction mixture containing an excess amount of ATP, the reaction mixture was heated at 80°C for 10 min and remaining butirosin activities were measured on paper discs and by thin layer chromatography. The results are shown in Fig. 7. Butirosin was inactivated by cell free extracts of *B. vitellinus*, *E. coli* C600 (pTABR10) and *B. subtilis* RM125 (pTABR10). The level of inactivation by the cell free extract of *B. vitellinus* is lower than those by cell free extracts of *E. coli* and *B. subtilis* carrying pTABR10, respectively. This suggests that butirosin-inactivating enzyme ac-

FIG. 8. Assay of Aminoglycoside 3'-Phosphotransferase Activity (II).

Aminoglycoside-inactivating activities in cell-free extracts were measured with neomycin (Nm), kanamycin A (Km A), kanamycin B (Km B) and tobramycin (3'-deoxy Km B) as substrates. Inactivation was not observed when tobramycin was used as a substrate. This means that the enzymatic inactivating site is the 3'-OH residue of antibiotics.

FIG. 9. Confirmation of the Phosphorylation of Aminoglycoside by Cell Free Extracts.

(A) Butirosin was treated with cell free extracts with or without addition of ATP (phosphoryl donor). Inactivation was observed only when ATP was added to the reaction mixture. (B) Inactivated samples of butirosin from the reaction (A) with ATP were treated with bacterial alkaline phosphatase (Boehringer) at 37°C for 60 min. The antibiotic activity was recovered with the phosphatase treatment. (C) Neomycin (Nm), kanamycin A (Km A) or kanamycin B (Km B) inactivated by cell free extracts of *B. subtilis* RM125 (pTABR10) or *E. coli* C600 (pTABR10) was treated with alkaline phosphatase. The treatment restored the antibiotic activities.

tivity in *E. coli* and *B. subtilis* increased due to the gene dosage effect.

To confirm phosphorylation of the antibiotics at the 3'-position, several aminoglycoside antibiotics were used as substrates. Figure 8 shows that tobramycin (3'-deoxykanamycin B) was not inactivated by cell free extracts from *B. vitellinus*, *E. coli* (pTABR10)

and *B. subtilis* (pTABR10). This indicated that the aminoglycoside inactivating enzymes in both *E. coli* and *B. subtilis* were modifying 3'-OH residues of aminoglycosides.

Inactivation required ATP and the inactivated aminoglycosides recovered their activities on treatment with bacterial alkaline phosphatase (Fig. 9). These experimental data

show that recombinant plasmid pTABR10 really expresses its aminoglycoside-3'-phosphotransferase gene from *B. vitellinus* both in *E. coli* and *B. subtilis*.

DISCUSSION

In this paper, we described the cloning and functional expression of the aminoglycoside 3'-phosphotransferase gene derived from *B. vitellinus* both in *E. coli* and *B. subtilis* by using pTA1302 as a vector. This indicates the utility of our plasmid pTA1302 as a shuttle vector in both *E. coli* and *B. subtilis*. But, in the case of cloning in *B. subtilis*, deletion of recombinant plasmids was often observed. Similar phenomena of deletion of recombinant plasmids were reported by various researchers (for instance, Kreft *et al.*¹⁴). In our case, pTABR10 is stably maintained in *B. subtilis*, but pTABR4 is not. pTABR4 and pTABR10 are constructed from the same components, pTA4', pBR322' and the 4.6 Md fragment of *B. vitellinus*. The only difference is the orientation of insertion of the 4.6 Md fragment at the *Hind*III site of pTA1302. If instability of pTABR4 depends on its orientation of insertion, interaction of the DNA sequence between the vector plasmid and donor DNA will play a great role in plasmid instability. Similar results were obtained when we constructed shuttle vector plasmids by *in vitro* ligation of staphylococcal plasmid pTP4 (Cm^r, 2.7 Md) and pBR322 by *Hind*III-ligase treatment. One of the shuttle vectors, pTA1312, was not stably maintained in *B. subtilis*, whereas pTA1311, which had the opposite orientation of ligation

compared to pTA1312, was fairly stable.

A butirosin-resistance gene has already been cloned by Courvalin *et al.*¹³ from a butirosin-producing bacterium, *B. circulans* NRRL B-3312. But, restriction patterns of the cloned fragments in our experiment were different from those of Courvalin *et al.*

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