New shuttle vectors for *Escherichia coli* and *Bacillus subtilis*.

II. Plasmid pHY300PLK, a multipurpose cloning vector with a polylinker, derived from pHY460

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ABSTRACT

In vivo and *in vitro* recombination techniques were used to construct a new cloning vector, pHY300PLK (4.7 kb) from the shuttle vector pHY460 (7 kb). The newly derived shuttle vector can replicate and express the tetracycline resistance gene (Tc^R) in both *Escherichia coli* and *Bacillus subtilis*. pHY300PLK contains the Tc^R gene, the ampicillin resistance gene (Ap^R), two replication origins for *E. coli* and *B. subtilis* and a polylinker derived from π AN7. The unique cloning sites are *Bal*I, *Bam*HI, *Ban*I, *Bgl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*I, *Sal*I, *Sma*I, *Pvu*I and *Xba*I. pHY300PLK is characterized as a copy-number mutant in *E. coli*. (**Key words:** shuttle vector; *B. subtilis*; tetracycline; ampicillin; copy-number mutant; incompatibility)

1. INTRODUCTION

At the first step of the gene manipulation *in vitro*, one must choose a good host-vector system for a desired foreign DNA. Many excellent cloning vectors in *E. coli*, are available. However, the expression of foreign DNA is often difficult in *E. coli*, and new host-vector systems need to be developed. Powerful gene manipulation systems have been developed in *E. coli*, so that bifunctional vectors between *E. coli* and other organisms are especially useful. Recently several bifunctional vectors have been developed. These include *Bacillus subtilis* (Ishiwa and Tsuchida 1984; Ostroff and Péne 1984 a, b), *Streptococcus sanguis* (Dao and Ferretti 1985), yeast (Broach *et al.* 1979; Heusterspreute *et al.* 1985) and several eukaryotic vectors (Gluzman 1982).

In the present communication we describe newly constructed shuttle vector, pHY300PLK (4.7 kb) derived from the shuttle vector pHY460 (7 kb). (1) pHY300PLK is one of the smallest shuttle vectors having two selective markers. (2) pHY300PLK is shown to be a copy-number mutant in *E. coli*, and can produce a large amount of plasmid DNAs in small culture. (3) In a *E. coli* *rec*⁺ strain, the oligomer form DNAs are produced, which are effective

Abbreviations: *Ap*, ampicillin; *kb*, kilobase pairs; *Km*, kanamycin; *MIC*, minimum inhibitory concentration; *ori*, origin of DNA replication; ^R, resistance; ^S, sensitivity; *Tc*, tetracycline; [], indicates plasmid-carrier state.

in transforming competent cells of *B. subtilis* (Canosi *et al.* 1978; de Vos *et al.* 1981).

2. MATERIALS AND METHODS

(a) *Bacterial strains and plasmids*

The *E. coli* K12 strain C600 F⁻ *thi-1 thr-1 pro leuB6 lacY1 tonA21 supE44 hsdR hsdM* λ^- (Maniatis *et al.* 1982), the *B. subtilis* strain UOT0277 *hisA1 metB5 recE4 nonB1* (Shimotsu *et al.* 1983) and plasmid vector pACYC177 (Chang and Cohen 1978) were provided by H. Saito and H. Yoshikawa (University of Tokyo). π AN7 DNA (B. Seed, unpubl.) was provided by T. Sako (Yakult Institute).

(b) *Cell growth*

L-broth (Lennox 1955) was employed for routine cultivation of *E. coli* and *B. subtilis*.

(c) Preparation of plasmid DNA and transformation procedures of *E. coli* followed Maniatis *et al.* (1982) with minor modifications.

(d) *Transformation procedures of Bacillus subtilis*

The competent cells were prepared by the methods described in Sadaie and Kada (1983) with some modifications. When the cells had grown to the end of log phase in the first culture, 50% of glycerol was added to the culture to the final concentration of 12.5%. A 0.4 ml aliquot of the culture was dispensed into small plastic tubes and quickly frozen in a methanol-dry ice bath. The frozen cells were kept at -80°C until use. The competent cells in the second culture were prepared from the frozen culture. The frozen culture was liquefied at 37°C , the cells were harvested by centrifugation and then resuspended into 3 ml of fresh SPII medium. The competent cells were obtained after 90 min of cultivation. The number of transformants were scored on L-agar containing 30 μg of tetracycline per ml. By using frozen cells, we could work rapidly and obtain highly reproducible results for more than six months.

(e) *Enzymes*

Restriction endonucleases were purchased from Boehringer-Mannheim (FRG), Bethesda Research Laboratories (Maryland, USA), New England Biolabs (Massachusetts, USA), Takara Shuzo (Kyoto, Japan), and Toyobo Co. Ltd., (Osaka, Japan). Enzymatic reactions were carried out under the conditions recommended by the suppliers. T4 DNA ligase, T4 DNA polymerase, *Hind*III linker were from Takara Shuzo and experimental conditions were followed by Maniatis *et al.* (1982).

3. RESULTS AND DISCUSSION

Construction of shuttle vector pHY300PLK: In the previous paper (Ishiwa and Tsuchida 1984), we reported a shuttle vector pHY460 (7 kb) capable of replicating in both *E. coli* and *B. subtilis* from a fused plasmid of pACYC177 (Ap^R Km^R) of *E. coli* and pAM α 1 (Tc^R) (Clewell *et al.* 1975) of *Streptococcus faecalis*. However, it was not always stable in *B. subtilis* when carrying foreign DNA. Therefore, in an attempt to stabilize the vector we constructed several deletion plasmids from pHY460 as shown in Fig. 1. These plasmids

Fig. 1. Schematic diagrams of the family of pHY plasmids. Only those restriction sites are shown which are relevant for the construction. Functionally important regions are indicated as Ap^R , Tc^R , ori-pAM α 1, ori-177 and PLK (polylinker).

retain main functional regions containing the replication origins of pAM α 1 (*ori*-pAM α 1) and pACYC177 (*ori*-177), as well as the Tc^R gene and the Ap^R gene. In addition, the polylinker region from π AN7 was inserted into the smallest shuttle vector to get pHY300PLK (4.7 kb). pHY300PLK should be more suitable as a shuttle vector for use in both *E. coli* and *B. subtilis*. In the following, we will give some detailed descriptions about pHY300PLK (Fig. 1).

(a) *Isolation of deletion plasmids in vivo from pHY460*: In the course of a stability test of pHY460 in *B. subtilis*, 48 clones were cultivated for about 50 generations in L-broth with 10 μ g/ml of Tc, and then the molecular size of the plasmids extracted from each culture was measured by agarose gel electrophoresis. Among them a deletion plasmid with molecular size of about 3.85 Md (5.9 kb) was isolated incidentally and named pHY385. pHY385 had lost the unique restriction sites of *Xba*I, *Ava*I and *Bam*HI in pHY460 (Fig. 1). pHY385 DNA thus extracted from *B. subtilis* cells was able to transform *E. coli* cells to Tc^RAp^R.

(b) *Construction of deletion plasmids in vitro*: The short *Hae*II fragment between the *Hae*II¹ and *Hae*II² sites was eliminated from pHY385. The new plasmid of about 3.4 Md (5.2 kb) was named pHY340; it was found to have the unique *Hae*II site.

To reduce the molecular size further and to introduce a polylinker into pHY340 vector, the following manipulations were performed. Since plasmid pHY340 contains two *Eco*RI sites (see *Eco*RI¹ and *Eco*RI² in Fig. 1), the circular plasmid DNA was cleaved with *Eco*RI under a condition allowing cleavage of the molecules at only one of the two sites. The full length linear DNA was purified by low-melting-point agarose gel electrophoresis and then treated with T4 DNA polymerase to fill in the cohesive ends. The DNA was ligated to the circular form, and used to transform *E. coli* to Tc^RAp^R. One of the plasmids from four clones tested as such had the unique *Eco*RI site and named pHY339.

To insert a *Hind*III linker into the *Sac*II site of pHY339, plasmid pHY339 was digested with *Sac*II, treated with T4 polymerase to fill in the cohesive ends, ligated with *Hind*III linkers and used to transform *E. coli* to Tc^RAp^R. A plasmid which generated *Hind*III sites but had lost the *Sac*II site was chosen. In order to keep a single *Hind*III site at the ligation point, it was treated with *Hind*III to remove the excess *Hind*III linkers, circularized with T4 DNA ligase, and amplified in *E. coli*. The new plasmid was named pHY 336. The polylinker region having a *Eco*RI site at one terminus and a *Hind*III site at the other terminus derived from π AN7 (Seed, B. unpubl.) was inserted into pHY336 DNA at the sites of *Hind*III and *Eco*RI by ligation, and the

Fig. 2. The physical and genetic map of *pPHY300PLK*. This plasmid specifies *Tc^R* and *Ap^R* in *E. coli*, and the origins of replication are designated *ori-177* and *ori-pAMα1*.

recombinant DNA was used to transform *E. coli* cells to *Tc^RAp^R*. These processes are shown in Fig. 1. One of the plasmids with a molecular weight of about 3 Md (4.7 kb) containing a polylinker region (*HindIII*-*XbaI*-*BglII*-*PstI*-*SalI*-*BamHI*-*SmaI*-*EcoRI*) was chosen and named *pPHY300PLK*.

Restriction map of *pPHY300PLK*: A restriction map of *pPHY300PLK* (Fig. 2) was made from double and triple endonuclease digests of the plasmid DNA

with help of pHY460 and its derivatives (Ishiwa and Tsuchida 1984). pHY300PLK was found to have the unique substrate sites for *Bal*I, *Bam*HI, *Ban*I, *Bgl*I, *Bgl*II, *Bst*EII, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*I, *Sal*I, *Sma*I, *Pvu*I and *Xba*I. The dual substrate sites were for *Acc*I, *Nco*I, *Sca*I, *Pst*I and *Pvu*II. The following restriction sites were lacking; *Ava*I, *Apa*I, *Ban*II, *Bbe*I, *Bcl*I, *Kpn*I, *Mul*I, *Nru*I, *Sac*I, *Sla*I, *Stu*I and *Tth*111I.

Replication and expression of the Tc^R gene of pHY300PLK in both E. coli and B. subtilis: The plasmid DNA of pHY300PLK extracted from *E. coli* [pHY300PLK] was used to transform *B. subtilis* UOT0277. All of twelve Tc^R transformants contained plasmids with a size of about 3 Md (4.7 kb). After shuttling between *E. coli* and *B. subtilis*, the molecular integrity of pHY300PLK DNA had well been conserved as determined based on the observations of molecular sizes and phenotypes.

MIC values, a mutation that affects for incompatibility and copy-number in E. coli: The minimum inhibitory concentration (MIC) values of Tc were determined by the tube dilution test method (Malke and Holm 1981) for strains of *E. coli* and *B. subtilis* which carried pHY340 or pHY300PLK. In the case of *E. coli*, *E. coli* [pHY300PLK] expressed a higher MIC value than *E. coli* [pHY340]. *E. coli* [pHY300PLK] was able to grow at the concentration 75 µg/ml of Tc but not at 100 µg/ml of Tc in L-broth. It means that the MIC value of Tc for *E. coli* [pHY300PLK] should be between 75 and 100 µg/ml. Thus, we put MIC=100 µg/ml tentatively. Similarly, the MIC value of *E. coli* [pHY340] was between 25 and 30; then MIC=30 µg/ml.

We have experienced a relatively higher plasmid yield from *E. coli* [pHY300PLK] than *E. coli* [pHY340] in a plasmid isolation procedure throughout the course of the present experiments. Therefore it was suggested that a higher MIC value of *E. coli* [pHY300PLK] compared with that of *E. coli* [pHY340] could be explained in terms of gene dose effect due to a copy-number mutation in the RNA I region of the *ori*-177 region. Actually, the nucleotide sequence analysis of pHY300PLK (H. I., in preparation) has revealed that there is a single base substitution in the regulatory RNA I when compared with the *ori*-p15A (Selzer *et al.* 1983). Note that pACYC177 was derived from the p15A replicon (Chang and Cohen 1978). The whole sequence of pHY300PLK will be published elsewhere.

Copy number mutations in the RNA I region usually have altered incompatibility and vice versa in ColEI as reviewed by Davison (1984). This could be the case in the present mutation found in pHY300PLK. Double transformation experiments described below clearly showed that the nature of incompatibility of pHY300PLK was changed to be compatible. First, pACYC177 (Ap^RKm^R) was able to transform *E. coli* [pHY300PLK (Tc^RAp^R)] to Tc^RAp^R

Km^R. Furthermore, DNAs extracted from double transformants showed two species of molecules corresponding to each plasmid. Secondly, in a series of transformation experiments in the reverse direction, no transformants were obtained. These results suggest that functional RNA I produced by pACYC 177 repressed the initiation of DNA replication of pHY300PLK which was introduced later. The segregation test (Tomizawa and Itoh 1981) has not been done for doubly transformed bacteria.

As in the case of pACYC177 reported by Chang and Cohen (1978), pHY300 PLK was found to undergo only limited amplification in the presence of chloramphenicol (Cm). However, we can easily obtain a large amount of the plasmid DNA and its derivatives in *E. coli* by cultivating them in L-broth overnight at 37°C without an addition of Cm.

MIC values for B. subtilis: MIC values of Tc for *B. subtilis* UOT0277 carrying pHY340 or pHY300PLK were also determined in a similar way to that for *E. coli*. *B. subtilis* [pHY340] grew at 150 µg/ml of Tc in L-broth with aeration but not at 200 µg/ml; thus MIC was estimated to be 200 µg/ml. *B. subtilis* [pHY300PLK] grew at 100 µg/ml of Tc but not at 150 µg/ml (MIC = 150). The plasmid-free strain of *B. subtilis* was sensitive to Tc and its MIC value was lower than 12.5 µg/ml in this study. In contrast to the case of *E. coli*, MIC value of *B. subtilis* [pHY300PLK] turned out to be slightly smaller than that of *B. subtilis* [pHY340]. This difference of MIC values was reproducible and should be meaningful. From the nucleotide sequence analysis of the Tc^R determinant in pHY300PLK (H.I., in preparation), the terminal codon of a long open reading frame for Tc^R was found about 50 bases upstream of the *EcoRI* site, and a possible terminator sequence with a hairpin structure (Rosenberg and Court 1979) was discovered between the terminal codon and the *EcoRI* site (Fig. 3). In the process of construction (Fig. 1), the region of about 700 bp between the *EcoRI*¹ site and the *sacII* site in pHY340 had been

Fig. 3. Terminator sequence of Tc^R determinant originated from pAMα1. The anti-sense strand for the terminator region between *HincII* and *EcoRI* sites is shown. The inverted repeat sequences are underlined, and the T residues are found (Rosenberg and Court 1979).

deleted in pHY300PLK, which made up most of the differences around the Tc gene between the two vectors. Thus, it is suggested that due to this deletion *B. subtilis* [pHY300PLK] showed slightly lower resistance to Tc. If this is the case, there might exist some signal which is functional only in *B. subtilis* for enhancing the expression of the Tc^R gene within the 700 bp region. To clarify this point further we need more experimental data.

A successful system for transformation: In contrast to the report by Chang and Cohen (1978), pACYC177 and a series of pHY plasmids in our study formed multimers about 30% in the *rec*⁺ strain but very rarely in the *recA* strain of *E. coli*. Only the oligomer form DNAs could be transformed into *B. subtilis* competent cells with high efficiency (Canosi, *et al.* 1978; de Vos *et al.* 1981). Accordingly, the shuttle vector DNA which is amplified in the *rec*⁺ strain of *E. coli* is suitable as a donor DNA for transforming *B. subtilis* competent cells. Crude DNAs extracted from a small scale culture of a *E. coli rec*⁺ strain are sufficient enough to use for transformation into both *E. coli* and *B. subtilis*. In addition, frozen competent cells of *B. subtilis* processed by our method have continued to give good transformation results for more than six months and thus are recommended to use. By using the shuttle vector, pHY 300PLK amplified in any *E. coli rec*⁺ strain with frozen competent cells, the transformation procedure for *B. subtilis* has become just as easy as for *E. coli*.

In conclusion, we have established a new shuttle vector "system", which is useful for gene cloning in *E. coli* and *B. subtilis* simultaneously.

pHY300PLK is one of the smallest shuttle vectors so far available. However, pHY300PLK shortened even further, while retaining some of functionally essential regions. The result will be published elsewhere.

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