*Gene*, 2(1977) 75—93



# CONSTRUCTION AND CHARACTERIZATION OF NEW CLONING VEHICLES
# I. AMPICILLIN-RESISTANT DERIVATIVES OF THE PLASMID pMB9

(Plasmid vectors; molecular cloning; tetracycline resistance transposon; restriction enzyme mapping)

FRANCISCO BOLIVAR*, RAYMOND L. RODRIGUEZ* , MARY C. BETLACH and HERBERT W. BOYER

*Department of Biochemistry and Biophysics, University of California, San Francisco, Calif. 94143 (U.S.A.)*

(Received January 31st, 1977)
(Accepted June 23rd, 1977)

SUMMARY

In vitro recombination via restriction endonucleases and the in vivo genetic translocation of the Ap resistance ($Ap^r$) gene resulted in the construction of a new cloning vehicle, the plasmid pBR313. This vector was derived from a ColE1-like plasmid and, while it does not produce colicin E1, it still retains colicin E1 immunity. The $Ap^r$ and tetracycline resistance ($Tc^r$) markers carried in pBR313 were derived from the ampicillin transposon (TnA) of pRSF2124 and pSC101 respectively. During the construction of pBR313, the TnA component was altered and the $Ap^r$ gene in pBR313 can no longer be translocated.

This plasmid has a molecular weight of 5.8 Mdalton and has been characterized using thirteen restriction enzymes, six of which (*Eco*RI, *Sma*I, *Hpa*I, *Hin*dIII, *Bam*HI and *Sal*I) cleave the plasmid at unique restriction sites. This allows the molecular cloning of DNA fragments generated by these six enzymes. The restriction sites for the latter three enzymes, *Hin*dIII, *Bam*HI and *Sal*I, are located in the $Tc^r$ gene(s). Cloning DNA fragments into these sites alters the expression of the $Tc^r$ mechanisms thus providing a selection for cells carrying recombinant plasmid molecules. An enrichment method for $Ap^rTc^s$ cells carrying recombinant plasmid molecules is described.

*Present addresses: (F.B.)Departamento Biologia Molecular, Instituto de Investigaciones Biomedicas, Universidad Nacional Autonoma de Mexico, Mexico 20, D.F.; (R.L.R.) Department of Genetics, Briggs Hall, University of California, Davis, CA 95616 (U.S.A.).

Abbreviations: Ap, ampicillin; Cyc, cycloserine; Tc, tetracycline.

## INTRODUCTION

There are several critical components which facilitate recombinant DNA research. The ease with which recombinant DNA research can proceed will in part depend on the improvement of one of these components, the cloning vehicle. It is now clear that bacterial plasmids, bacteriophages and animal viruses can serve as vectors for cloning DNA fragments. Two bacterial plasmids used in most of the initial cloning experiments as cloning vectors are pSC101 (Cohen et al., 1973) and ColE1 (Hershfield et al., 1974). Other cloning vectors have been derived from these two plasmids (Hamer and Thomas, 1976; Hershfield et al., 1976; So et al., 1976) which improve on their utility.

We have undertaken the development of a series of plasmids with the goal of obtaining a set of multipurpose cloning vehicles. In this paper, we describe the construction of plasmid pBR313 with the ColE1 mode of replication and which contains the genetic potential for resistance to the antibiotics ampicillin (Ap) and tetracycline (Tc). A tentative restriction map has been determined for this plasmid and its cloning properties have been characterized. pBR313 permits molecular cloning with DNA fragments derived by endonucleolytic action of the following restriction endonucleases: *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*I, *Hpa*I and *Sma*I. A procedure for selecting transformants which contain recombinant plasmids has been developed.

## MATERIALS AND METHODS

### *(a) Bacterial strains*

The following derivatives of *E. coli* K12 were used as recipient cells in transformation experiments: HB101 $F^-$ *pro leu thi lac*Y *Str*$^r$ $r_K^-m_K^-$ Endo$I^-$, *recA*$^-$ (Boyer and Roulland-Dussoix, 1969); RR1 $F^-$ *pro leu thi lac*Y *Str*$^r$ $r_K^-m_K^-$; Endo$I^-$. The *E. coli* B strain HB50 (*pro leu try his arg met thr gal lac*Y *Str*$^r$ $r_K^-m_K^-$) was used to prepare unmethylated plasmid DNA for *Eco*RII digestion (Yoshimori et al., 1972). The bacterial plasmids pRSF2124 and pSC101 in the *E. coli* K12 C600 background ($F^-$ *leu thi thr lac*Y) were kindly provided by S. Falkow and S. Cohen, respectively. The plasmid ColE1 (JC411 $F^-$, *leu his arg met gal mal xyl thy lac*Y *Str*$^r$) was obtained from D. Helinski. The plasmids pMB8 and pMB9 were maintained in and prepared from HB101.

### *(b) Preparation of plasmid DNA*

Preparation of plasmid DNA by amplification in the presence of chloramphenicol (170 μg/ml) was performed according to Clewell (1972). The DNA was purified by a modification of the cleared lysate technique of Guerry et al., 1973. The cleared lysate was extracted with an equal volume of phenol and the aqueous phase precipitated with two volumes of cold ethanol. The resuspended DNA, in a 5 ml volume of A50 agarose buffer (500 mM NaCl, 50 mM Tris pH 8.0, 1 mM EDTA), was applied to a 25 cm ×

50 cm Bio-Gel A50 agarose column (Bio-Rad), and the first peak fractions as determined by $A_{260nm}$ were pooled and ethanol-precipitated. The plasmid DNA was further purified by dye-buoyant centrifugation in a CsCl-propidium diiodide gradient, equilibrated for 18 h at 36 000 rpm, 20°C, in a Spinco SW 50.1 rotor. Small molecular weight RNA contaminates the plasmid DNA at this stage.

The band of supercoiled DNA as visualized with UV light was collected and the propidium diiodide extracted by passing the DNA over a 1 cm × 4 cm Dowex (AG 50W-X8 Bio-Rad) column. The eluent was dialyzed against 10 mM Tris—HCl, 1 mM EDTA, pH 7.4, ethanol-precipitated and resuspended in 50 mM Tris—HCl, 10 mM NaCl, 1 mM EDTA pH 7.4. DNA concentrations were determined spectrophotometrically in the above buffer; $A_{260nm}$ of 1.0 = 50 μg DNA/ml (Padmanabhan and Wu, 1972).

Preparation of plasmid DNA for the rapid analysis of the restriction endonuclease digestion pattern was performed according to the procedure described by Meagher et al. (1977).

*(c) Enzymes*

*Eco*RI restriction endonuclease was purified according to the procedure of Greene et al. (1974). The restriction endonucleases, *Alu*I (Roberts et al., 1976), *Hae*II (Roberts et al., unpublished observations), *Hae*III (Roberts et al., unpublished observations), *Bgl*I (Wilson and Young, unpublished observations), *Bam*HI (Wilson and Young, 1975), *Eco*RII (Yoshimori et al., 1975), *Hinc*II (Landy et al., 1974), *Hin*dIII (Danna et al., 1973), *Pst*I (Smith et al., 1976), and *Sal*I (Arrand et al., 1976), were purified according to the procedure described by Heyneker et al. (1976). *Hpa*I (Gromkova and Goodgal, 1972) was obtained from BRL laboratories. T4 polynucleotide ligase, a gift from H. Heyneker, was purified according to the procedure of Panet et al. (1973). Colicin E1 was prepared from the strain JC411 as described by Schwartz and Helinski (1971). All restriction enzymes were stored at −20°C in 50% glycerol, 20 mM $KH_2PO_4$—$K_2HPO_4$ pH 7.0, 1 mM EDTA, 1 mM $NaN_3$, and 100 mM NaCl.

*(d) Preparation of various restriction endonuclease DNA fragments*

DNA fragments were generated with various restriction endonucleases by digesting from 0.1 to 5.0 μg of DNA in a 10 to 30 μl volume according to the following conditions for each endonuclease: *Bgl*I, *Alu*I, *Hae*II, and *Hae*III, 6 mM Tris—HCl pH 7.9, 6 mM β-mercaptoethanol, 6 mM $MgCl_2$; *Hpa*I, 50 mM Tris—HCl pH 7.5, 5 mM β-mercaptoethanol, 5 mM $MgCl_2$, 50 mM NaCl; *Sma*I, 15 mM Tris—HCl pH 9, 6 mM $MgCl_2$, 15 mM KCl; *Bam*HI, 100 mM Tris—HCl, 5 mM $MgCl_2$ pH 7.6; *Eco*RI, 100 mM Tris—HCl, 5 mM $MgCl_2$, 100 mM NaCl, 0.02% NP40 (Particle Data Laboratories) pH 7.6; *Eco*RI*, 2 mM Tris—HCl, 2 mM $MgCl_2$, 20% glycerol pH 8.8; *Eco*RII, 100 mM Tris—HCl, 5 mM $MgCl_2$ pH 7.6; *Hinc*II, 100 mM Tris—HCl, 66 mM $MgCl_2$, 500 mM NaCl, 60 mM β-mercaptoethanol pH 8.0; *Hin*dIII, 6.6 mM Tris—HCl, 6.6 mM $MgCl_2$, 50 mM

NaCl, 7 mM β-mercaptoethanol pH 7.5; *Pst*I, 90 mM Tris—HCl, 10 mM $MgSO_4$ pH 7.5; *Sal*I, 8 mM Tris—HCl, 6 mM $MgCl_2$, 150 mM NaCl, 0.2 mM EDTA, 50 μg/ml bovine serum albumin. Endonuclease reactions were incubated at 37°C and stopped by heating to 63°C for 5 min or by the addition of 10 μl of 5% sodium dodecyl sulfate, 25% glycerol, 0.025% bromophenol blue (stop mixture).

*(e) Molecular weight standards*

For agarose gel electrophoresis, the following markers were used: the six *Eco*RI-generated fragments of the λ bacteriophage genome (13.7, 4.68, 3.7, 3.56, 3.03, and 2.09 md.); the *Eco*RI-generated linear forms of the plasmids pVH51 (2.2 md.) (Hershfield et al., 1976) and pMB8 (1.7 md.) and the six *Hin*dIII-generated fragments of the SV40 genome (1.13, 0.75, 0.68, 0.35, 0.28, 0.13 md.). For acrylamide gel electrophoresis the seven *Hin*dIII-generated fragments of the bacteriophage PM2 (3.5, 1.34, 0.6, 0.29, 0.26, 0.155, 0.07 md.) were used as molecular weight standards (Wes Brown, personal communication).

*(f) Ligation of DNA fragments*

The ligation reactions were incubated at 12°C in volumes ranging from 50 to 1000 μl containing 66 mM Tris—HCl, 6.6 mM $MgCl_2$, 10 mM dithiothreitol (Sigma), 0.2 mM ATP pH 7.6 and varying amounts of DNA. The reaction was started by the addition of 0.5 to 5.0 μl of T4 polynucleotide ligase. DNA ligation was monitored by the electrophoresis of small aliquots of the reaction taken at intervals.

*(g) Agarose and acrylamide electrophoresis*

Slab gels containing 0.8 to 1.0% agarose (Seakem) were prepared by autoclaving the agarose for 10 min in Tris—EDTA—borate buffer (90 mM Trizma Base (Sigma), 2.5 mM $Na_2$ EDTA, 90 mM $H_3BO_3$ pH 8.2). After the gels were poured and allowed to solidify, samples containing from 0.2 to 1 μg of DNA were loaded onto the gels in a 20 to 40 μl volume per slot. Electrophoresis was performed at 130 to 150 V at room temperature for 2 h. Slab gels of 7.5% acrylamide (1 mm thickness) were prepared by mixing 2.4 ml of 10X Tris—EDTA—borate buffer with 15.6 ml of water, 6 ml of 28% acrylamide (Bio-Rad), 0.6% bisacrylamide and 0.12 ml of 10% $NH_4SO_3$. After degassing, TEMED (12 μml) (Bio-Rad) was added and the mixture poured immediately. Acrylamide electrophoresis was performed at 120 V for 2 h. After electrophoresis, all gels were soaked for 5 min in 4 μg/ml ethidium bromide solution and illuminated with a short-wave UV transilluminator (Ultraviolet Products, San Gabriel, Calif.). A yellow no. 9 Kodak Wratten gelatin filter and NP Type 55 Polaroid film were used with a MP-3 Polaroid camera to photograph the gels.

*(h) Transformation of E. coli*

Chloroform-sterilized DNA in a 100 μl volume was adjusted to a final concentration of 30 mM $CaCl_2$ and added to 0.2 ml of cells prepared for transformation by the procedure of Cohen et al. (1972). The transformation mixture was allowed to stand on ice for 1 h after which it received a 60 sec, 42°C heat pulse. The heat pulse was terminated by the addition of 5 ml of Luria broth. Transformed cells were plated immediately or allowed to proceed into the logarithmic phase of cell growth before plating.

RESULTS

*(a) Construction and characterization of pMB9*

The construction and characterization of a series of ColE1-like plasmid derivatives have been reported (Betlach et al., 1976). From one of these plasmids we derived a small plasmid which is similar to pVH51 (Hershfield et al., 1976). pMB8 has a molecular weight of $1.72 \times 10^6$ daltons, is immune to colicin E1, exhibits a ColE1 mode of DNA replication in terms of copy number and replication in the presence of chloramphenicol but does not form a detectable relaxation complex (Clewell and Helinski, 1969). Although pMB8 offers some advantages as a cloning vector (e.g., small size, good yields of DNA, low background of protein synthesis in minicell systems), it does not have a good selective marker or substrate sites for several of the commonly used restriction endonucleases other than *Eco*RI. We then constructed a composite plasmid which affords cloning with different restriction enzymes by incorporating into pMB8 some components of the pSC101 plasmid which can confer $Tc^r$ to the cell. This plasmid, pMB9, was constructed by ligating the products of an *Eco*RI* endonuclease (Polisky et al., 1975) digest of pSC101 DNA with an *Eco*RI endonuclease digest of pMB8 (Rodriguez et al., 1976).

The plasmid pMB9 has a molecular weight of $3.5 \cdot 10^6$ daltons and one substrate site for each of the following restriction endonucleases: *Eco*RI, *Hin*dIII, *Sal*I and *Bam*HI. The relative positions of these sites (Fig. 1) were determined by acrylamide gel electrophoresis of double and triple digestions of plasmid DNA with various restriction enzymes. Since pMB8 has no *Hin*dIII, *Bam*HI or *Sal*I sites, these sites should be associated with that pSC101 DNA fragment introduced in pMB9. This assumption is supported by the fact that these three sites are present in pSC101 in the same relative positions (data not shown) and that the molecular cloning of DNA into the *Hin*dIII, *Bam*HI and *Sal*I sites alters the $Tc^r$ mechanism (Hamer and Thomas, 1976; Rodriguez et al., 1976). Since DNA inserted into the *Eco*RI site of pMB9 does not alter the expression of the $Tc^r$ mechanism, we believe that this site lies outside the $Tc^r$ gene. However, cloning into the *Eco*RI site of pSC101 has been found to affect the level and inducibility of $Tc^r$ (Tait et al., 1977).

Although *Eco*RI-generated recombinant plasmids of pMB9 can be selected by virtue of their resistance to tetracycline, transformants with recombinant

plasmids constructed by insertion of DNA fragments generated by *Hin*dIII, *Bam*HI and *Sal*I digestion and incorporated into the respective restriction sites can only be selected by colicin E1 immunity. Since the action of colicin E1 is extremely dependent on the physiological state of the cell and is accompanied by a high frequency of spontaneous mutations to colicin tolerance (Nagel De Zwaig and Luria, 1967), we chose to introduce another selective marker into pMB9. This was accomplished by the genetic translocation of an ampicillin resistance marker ($Ap^r$) from pRSF2124 (So et al., 1976) to pMB9.

*(b) Construction of $Ap^r$-$Tc^r$ derivatives of pMB9*

The $Ap^r$ marker has been shown to be translocated on a $3.2 \cdot 10^6$ daltons sequence of DNA which has been termed TnA (Heffron et al., 1975). Translocation of the TnA from pRSF2124 to pMB9 was accomplished by cotransforming *E. coli* strain RRI with a total of 5 $\mu$g of supercoiled pMB9 and pRSF2124 DNA at a respective molecular ratio of 2 : 1. $Ap^r$-$Tc^r$ transformants which occurred at a frequency of $6 \cdot 10^{-6}$ after 5 generations were screened for plasmid DNA which gave a linear 6.7 mdalton plasmid molecule upon digestions with *Eco*RI. While the majority of $Ap^r$-$Tc^r$ transformants contained varying ratios of pMB9 and pRSF2124, approximately 20% of the total $Ap^r$-$Tc^r$ transformants give one linear plasmid DNA molecule upon *Eco*RI digestion. These *Eco*RI-generated linear molecules had molecular weights of $6.7 \cdot 10^6$ daltons which corresponds to the sum of the molecular weights of the TnA ($3.2 \cdot 10^6$ daltons) and pMB9 ($3.5 \cdot 10^6$ daltons). These plasmid molecules were shown to confer resistance to Ap and Tc when transformed back into *E. coli* RR1.

The presence of a single asymmetrically located *Bam*HI site about $1 \cdot 10^6$ daltons from the end of the TnA (Heffron et al., 1977) made it possible to localize the various TnA insertion sites in pMB9 (Fig. 1). In the case of two $Ap^r$-$Tc^r$ derivatives of pMB9, designated pBR312 and pBR26, mapping the relative positions of the restriction and TnA insertion sites was carried out by digestion with combinations of various restriction enzymes as shown in Plate I(a). In relation to the molecular weight standards (slots 4, 7 and 10) linear molecules of pBR312 and pBR26, having molecular weights of $6.7 \cdot 10^6$ daltons, were also generated by digestion with *Hin*dIII (data not shown).

*Bam*HI digests of pBR312 and pBR26 gave two fragments in each case with molecular weights of 5.1 and $1.6 \cdot 10^6$ daltons and 5.06 and $1.65 \cdot 10^6$ daltons, respectively. By following a *Bam*HI digestion of these plasmids with an *Eco*RI digest, the smallest band of pBR312 and the largest band of pBR26 were each cleaved by *Eco*RI to give a third band of $0.4 \cdot 10^6$ daltons. These results enable us to localize the TnA insertion counterclockwise to the *Eco*RI site in pBR312 and clockwise to the *Eco*RI site in pBR26. Since the smallest *Bam*HI fragments of pBR312 and pBR26 are less than $2.2 \cdot 10^6$ daltons, this indicates that the orientation of the TnA is such that the one-third portion which is known to carry the $Ap^r$ gene (Heffron et al., 1977) is proximal to the *Bam*HI site in the $Tc^r$ gene (Fig. 1).

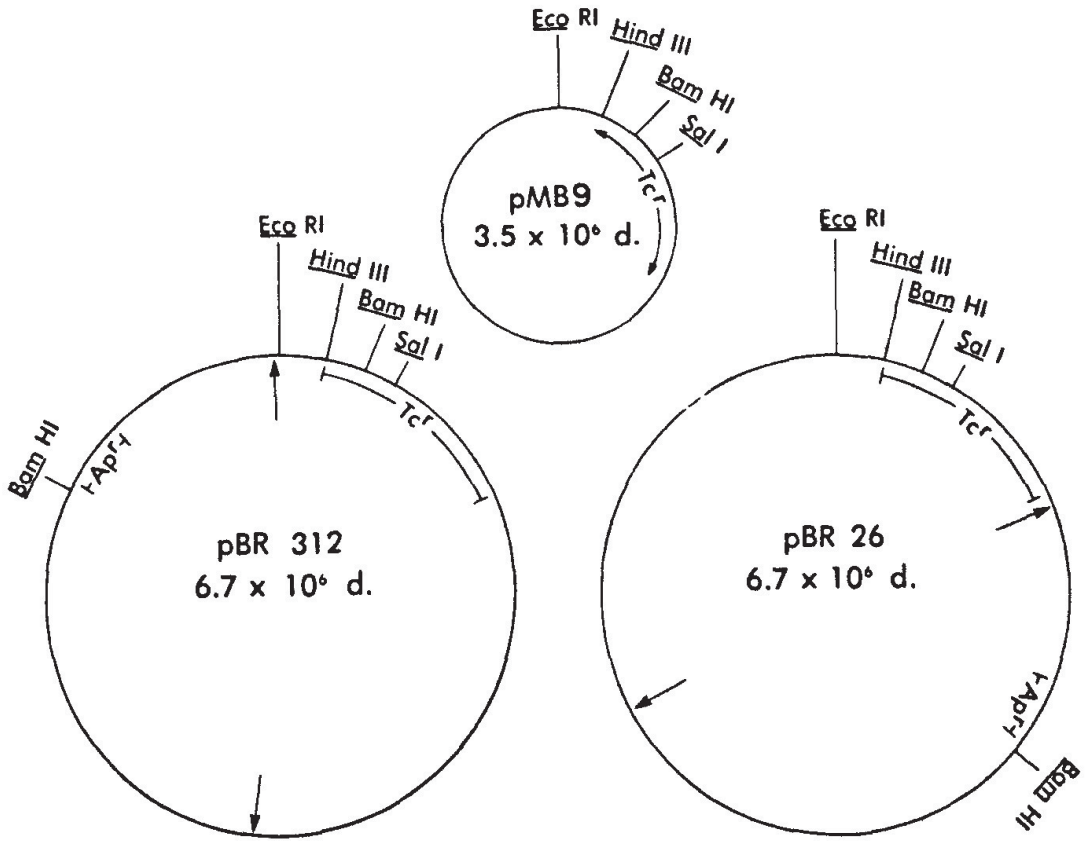



Fig. 1. Schematic representation of pMB9 DNA showing the $Tc^r$ region with the *Eco*RI, *Hind*III, *Bam*HI and *Sal*I sites. The arrows represent the position of the IR from TnA in two $Ap^r$ derivatives of pMB9: pBR312 and pBR26.

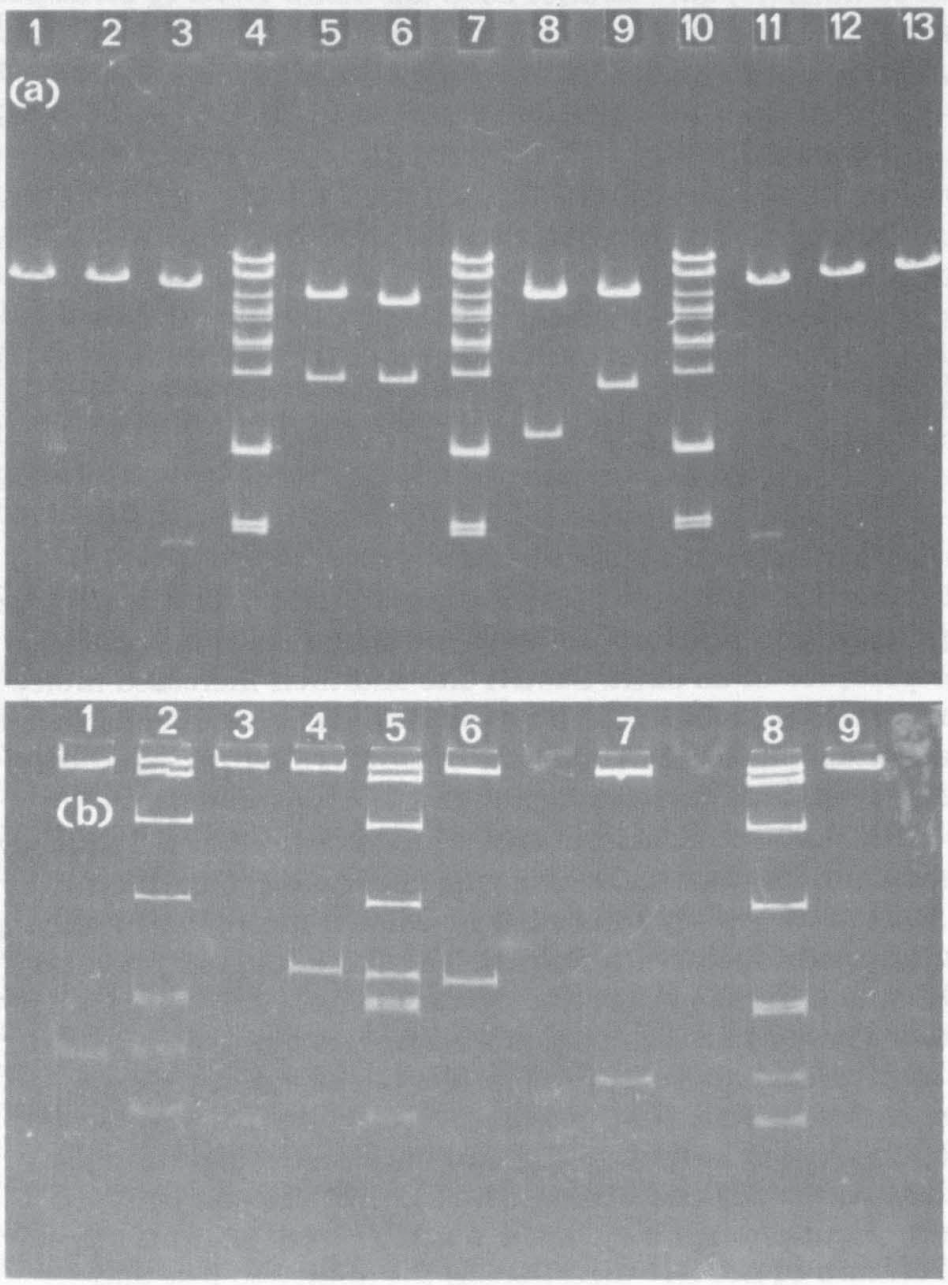
*(c) Construction and characterization of pBR313*

Although pBR312 and pBR26 now possess a second strong selective marker in the form of $Ap^r$, their potential usefulness as molecular cloning vectors is diminished by the presence of an extra *Bam*HI site and their increased molecular weights. Consequently, we decided to remove the *Bam*HI site contributed by the TnA and simultaneously reduce the size of pBR312 by partial *Eco*RI* digestion. After digestion, the DNA was ligated in a 500 $\mu$l volume and then transformed into RR1. $Ap^r$-$Tc^r$ transformants, which occurred at a frequency of $3 \cdot 10^{-7}$, were screened for plasmid DNA giving linear molecules upon *Bam*HI digestion. Out of 16 $Ap^r$-$Tc^r$ clones, 6 gave linear molecules of varying molecular weights after treatment with *Bam*HI. The three smallest plasmids (pBR313, pBR315 and pMB316) were selected for further study.

The molecular weight and relative position of restriction sites of pBR313 were determined as described above and the results of the acrylamide gel electrophoresis are shown in Plate I(b). The data from Plate I(b) have been summarized in Fig. 2a. As can be seen in Fig. 2b, the *Eco*RI* digestion has removed one of the two *Bam*HI sites from pBR313, pBR315 and pBR316 and reduced their molecular weights by 0.8, 1.5 and $1.4 \cdot 10^6$ daltons respectively. One useful feature of *Eco*RI* digestions is the ability to lose and

regenerate new *Eco*RI substrate sites. In the case of pBR316, the *Eco*RI site has been lost, whereas it is moved $0.16 \cdot 10^6$ daltons nearer to the *Hin*dIII site in pBR315 and even closer ($0.02 \cdot 10^6$ daltons) in pBR313 (Fig. 2b). This suggests that there are at least two *Eco*RI* sites, 0.16 and $0.02 \cdot 10^6$ daltons from the *Hin*dIII site in pMB9 which can be used to recreate new *Eco*RI substrate sites. It is also apparent from Fig. 2b that the segment of DNA between the *Hin*dIII and the *Sal*I sites of pBR313, pBR315 and pBR316, is unchanged after *Eco*RI* digestion as compared to pBR312, and pMB9.

It should be mentioned at this point that the estimated size of the $Ap^r$ and $Tc^r$ genes represented in Fig. 2 were determined indirectly on the basis of the reported values for the size of the TEM $\beta$-lactamase (Datta and Richmond,



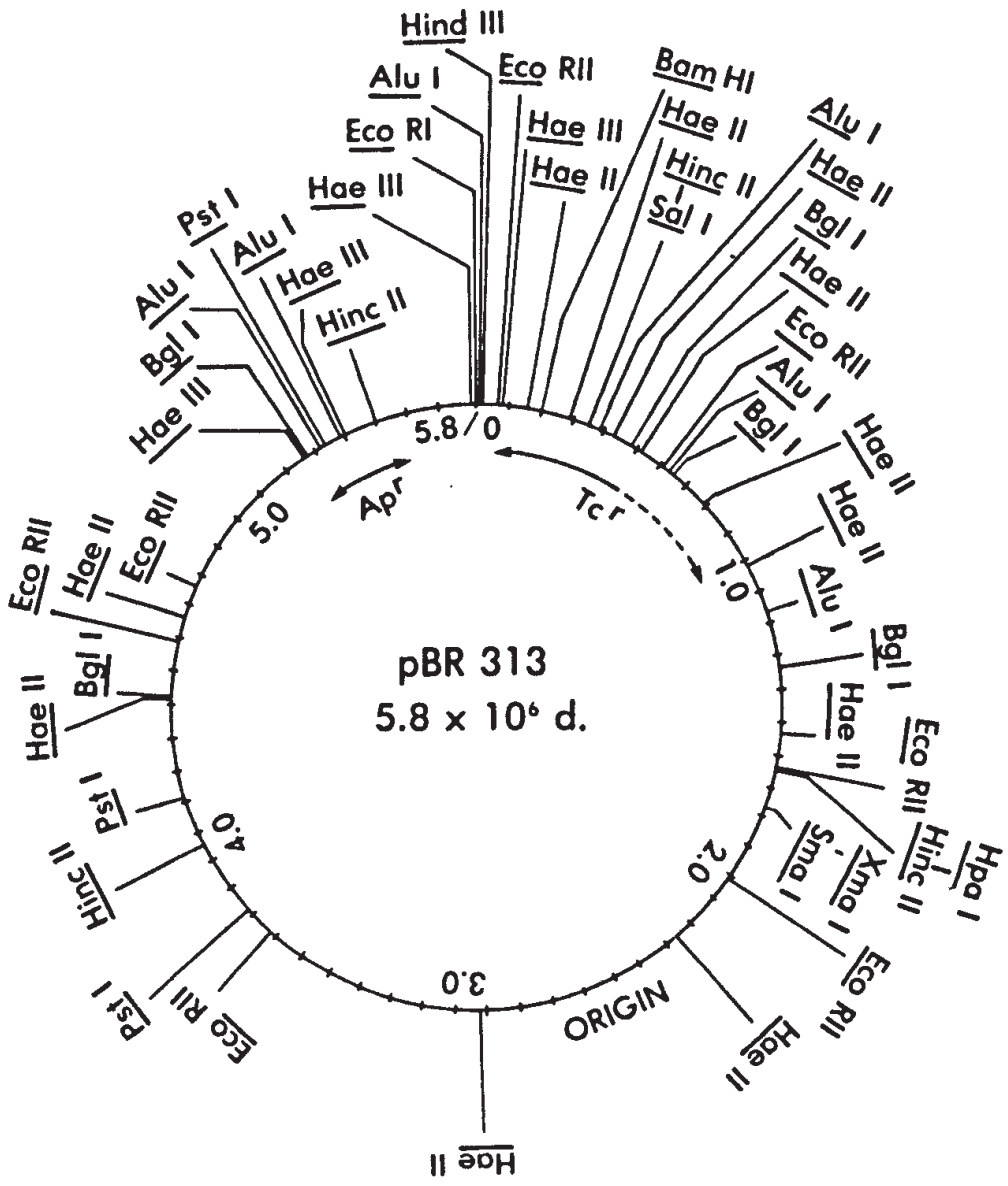
1966) and the $Tc^r$-associated proteins detected in the minicell system (Levy and McMurry, 1974; Tait et al., 1977). Positioning the left-hand boundary of the $Tc^r$ gene was based on our knowledge that cloning into the *Eco*RI site of pBR313 did not affect $Tc^r$ while cloning into the *Hin*dIII site did affect the expression of the $Tc^r$ mechanism. The position and size of the $Tc^r$ region is also consistent with the orientation of the TnA in pBR26. This follows from the consideration of the known position of the inverted repeats to the *Bam*HI site (Heffron et al., 1977). The $1.65 \cdot 10^6$ daltons *Bam*HI-generated fragment of pBR26 allows for $0.65 \cdot 10^6$ daltons of DNA between the pMB9 specified *Bam*HI site and the end of the $Tc^r$ gene(s) after accounting for the $1.0 \cdot 10^6$ dalton segment of the TnA.

The restriction endonuclease *Pst*I was used to further characterize pBR313. As shown in Plate II(a) (slot 5), pBR313 has three *Pst*I sites which give fragments of 0.4, 1.25 and $4.15 \cdot 10^6$ daltons. Since pMB9 was found to have no *Pst*I sites (Plate II(a), slot 2), it was concluded that the *Pst*I sites were associated with the TnA. This conclusion is further supported by the presence of five *Pst*I sites in pRSF2124 (Plate II(a), slot 3). Two of these five sites are known to be carried on the ColE1 portion of pRSF2124 (data not shown). It can also be seen that two of the three *Pst*I fragments present in pBR312 (Plate II(a), slot 4) 1.7 and $0.4 \cdot 10^6$ daltons, are present in pRSF2124, while only the smallest fragment is present in pBR313. From these results, we concluded that the $1.7 \cdot 10^6$ dalton fragment of pBR312 was reduced to $1.25 \cdot 10^6$ daltons in pBR313 by the *Eco*RI* digestion. The *Pst*I-*Eco*RI combination digest shown in slots 7, 8, and 9 of Plate II(a), corroborate the placement of the TnA counterclockwise to the *Eco*RI site in pBR312 (Fig. 1)

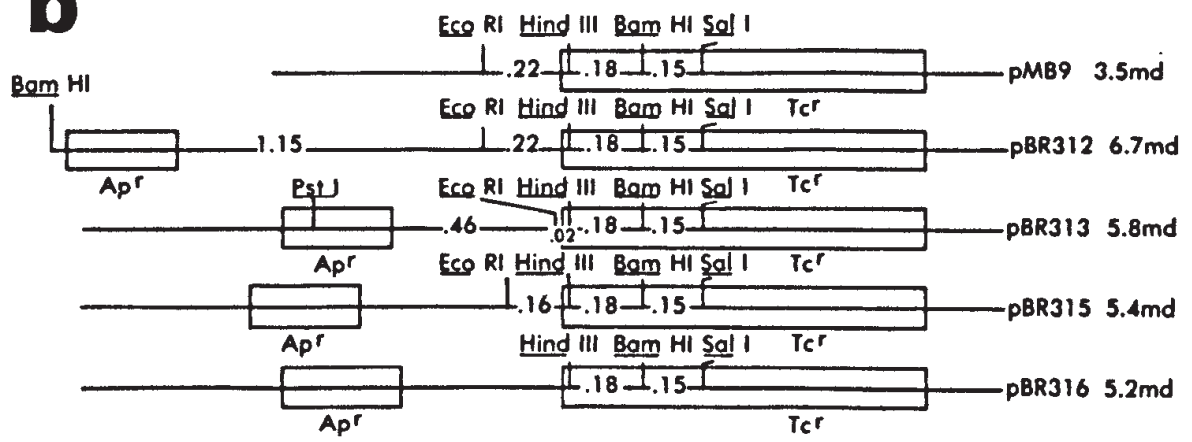
---

Plate I. (a) Agarose slab gel electrophoresis of plasmids pBR312 and pBR26 cleaved by *Eco*RI, *Bam*HI and *Sal*I endonucleases. Digested DNA (0.3 to 0.5 μg) was applied to the sample slots in 40 μl volumes. Agarose gel electrophoresis was carried out as described in MATERIALS AND METHODS. Molecular weight estimates are based on the 6 λ fragments generated by *Eco*RI, the linear forms of the plasmids pMB8 and pVH51 and the six *Hin*dIII-generated fragments of the SV40 genome (slots 4, 7, 10) (see MATERIALS AND METHODS). Slots 1, 2, and 5 show the *Eco*RI, *Sal*I and *Bam*HI digestions respectively of pBR26 plasmid DNA. Slot 3 shows the double digestion *Eco*RI-*Sal*I and slot 6 shows the *Eco*RI-*Bam*HI double digestions both in pBR26 DNA. Slots 9, 12, and 13 show the *Bam*HI, *Sal*I and *Eco*RI digestions respectively of pBR312 plasmid DNA. Slot 8 shows the *Eco*RI-*Bam*HI and slot 11 the *Eco*RI-*Sal*I double digestions of pBR312 DNA. For explanation see RESULTS (construction of $Ap^r$-$Tc^r$ derivatives of pMB9).
(b) Acrylamide slab gel electrophoresis of plasmid pBR313 DNA fragments obtained by double and triple digestions using *Eco*RI, *Hin*dIII, *Bam*HI and *Sal*I restriction endonucleases. Gel electrophoresis was carried out as described in MATERIALS AND METHODS. Molecular weight estimates are based on the (seven) *Hin*dIII-(generated) fragments of the PM2 phage genome. The restriction endonuclease digestion combinations for Fig. 3 are as follows: (Slot 1) *Eco*RI-*Bam*HI; (Slot 2) *Eco*RI-*Bam*HI-*Sal*I; (Slot 3) *Bam*HI-*Sal*I; (Slot 4) *Eco*RI-*Sal*I; (Slot 5) *Eco*RI-*Hin*dIII-*Sal*I; (Slot 6) *Hin*dIII-*Sal*I; (Slot 7) *Hin*dIII-*Bam*HI; (Slot 8) *Hin*dIII-*Bam*HI-*Sal*I; (Slot 9) *Eco*RI-*Hin*dIII. *Hin*dIII-digested PM2 markers are also present in slots 2 (bands 2, 3, 4, 5, 6, 8 and 10), slot 5 (bands 2, 3, 4, 6, 7, 8 and 9), slot 8 (bands 2, 3, 4, 5, 6, 8 and 10).

**a**


pBR 313
5.8 x 10⁶ d.
5.8 / 0
1.0
2.0
3.0
4.0
5.0
Ap^r
Tc^r
ORIGIN
Hind III
Eco RII
Bam HI
Alu I
Eco RI
Hae III
Hae II
Hinc II
Sal I
Bgl I
Pst I
Xma I
Sma I
Hpa I

**b**


Eco RI Hind III Bam HI Sal I
Bam HI
pMB9 3.5md
pBR312 6.7md
pBR313 5.8md
pBR315 5.4md
pBR316 5.2md
Ap^r
Tc^r
Pst I
.22
.18
.15
1.15
.46
.02
.16

since the *Pst*I-*Eco*RI-generated fragment ($0.6 \cdot 10^6$ daltons) was produced by cleaving the largest *Pst*I fragment carrying the $Tc^r$ gene. The position of the *Eco*RI site in pBR313 is localized at 0.46 mdaltons from one of the *Pst*I sites.

A derivative of pBR313 containing two *Pst*I sites (pBR317) was constructed by transforming RR1 with ligated *Pst*I-generated fragments of pBR313 and selecting for $Ap^r$ and $Tc^r$. This plasmid was found to lack the $0.4 \cdot 10^6$ dalton *Pst*I fragment seen in slot 5 of Plate II(a). After screening the plasmid DNA of a number of $Ap^r$-$Tc^r$ transformants, it was noticed that the $1.25 \cdot 10^6$ dalton *Pst*I fragment was present in all $Ap^r$-$Tc^r$ plasmids. Since this *Pst*I fragment was also present in some $Ap^s$-$Tc^r$ transformants, we believe that one orientation of this fragment is associated with the $Ap^r$ phenotype. Consequently, this places one *Pst*I site in the $Ap^r$ gene. Another $Ap^sTc^r$ plasmid (pBR318) was found to lack both 0.4 and 1.25 mdalton *Pst*I fragments present in pBR313 (data not shown).

*(d) Characterization of pBR313 by EcoRII digestion*

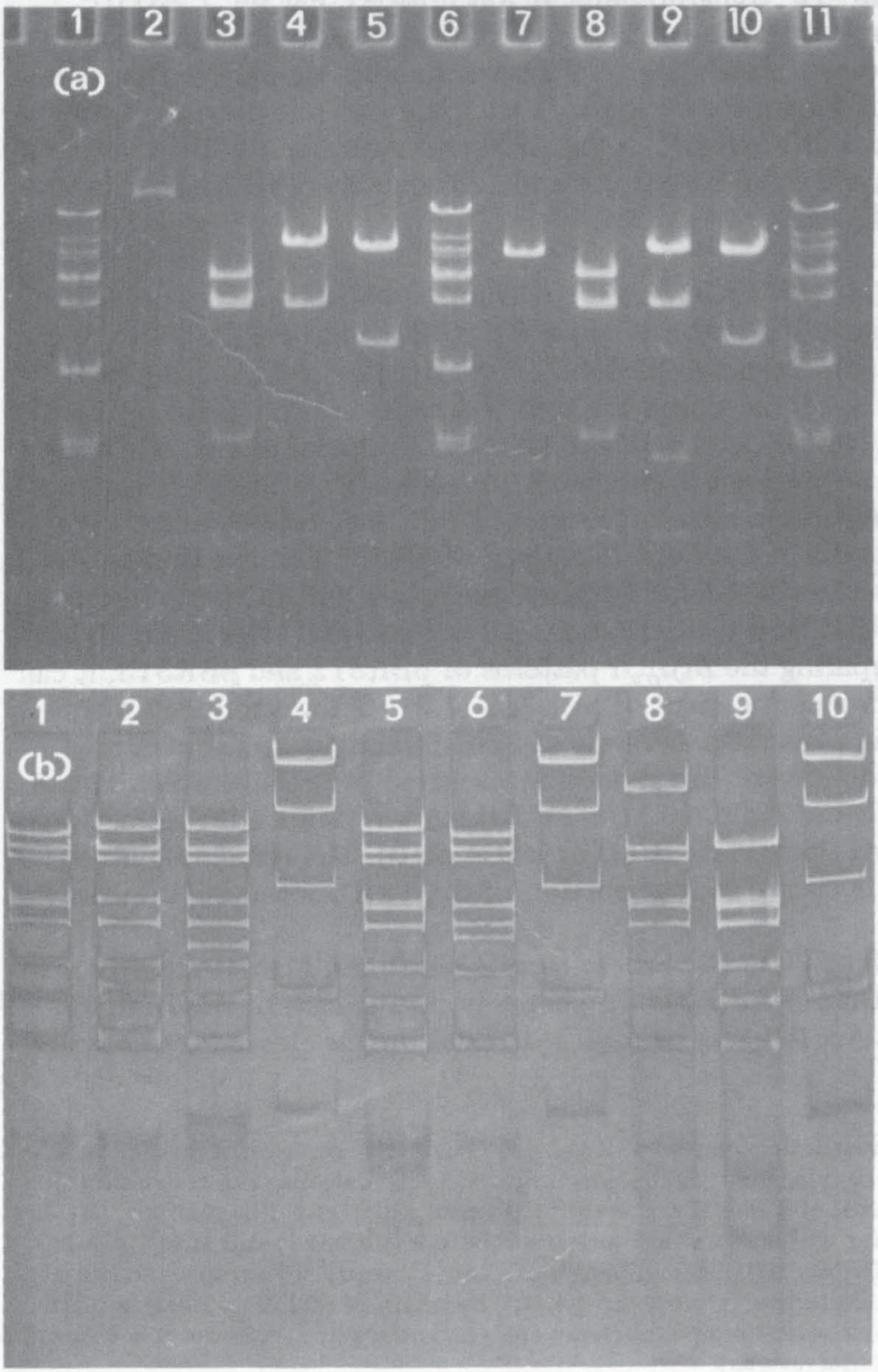
The *Eco*RII restriction patterns of pMB9, pBR312, pBR313, pBR316 are shown in Plate II(b). As shown in slot 9 (Plate II(b)), *Eco*RII produces at least 9 fragments in pMB9. Some of these fragments are common to pBR312 (slot 8), pBR316 (slot 6), and pBR313 (slot 5). As the largest *Eco*RII fragment of pMB9 is not present in the *Eco*RII pattern of pBR312, we assume that the TnA was inserted into this piece of DNA. As a consequence, four new fragments appear in the *Eco*RII pattern of pBR312 which are not seen in pMB9. Combination *Pst*I-*Eco*RII digestions reveal that the two largest fragments of pBR312 and pBR313 contain the three *Pst*I sites of the TnA (Fig. 2a). By comparing the *Eco*RII patterns of pBR312 and pBR313, it can be seen that the largest fragment of pBR312 ($1.9 \cdot 10^6$) was reduced by $0.8 \cdot 10^6$ daltons in pBR313 as a result of *Eco*RI* activity. As shown in Fig. 2b, $0.2 \cdot 10^6$ daltons of this reduction occurred between the *Eco*RI and *Hin*dIII sites of pBR312 while $0.14 \cdot 10^6$ daltons was removed from between the *Eco*RI and *Pst*I sites (Plate II(a), slots 9 and 10). The remaining $0.46 \cdot 10^6$ dalton reduction occurred in that portion of the TnA containing the *Bam*HI site.

Slots 1, 2 and 3 of Plate II(b) show *Hin*dIII, *Sal*I and *Bam*HI digestions of *Eco*RII-digested DNA of pBR313 respectively. In the case of the *Hin*dIII digest, the largest *Eco*RII fragment ($1.1 \cdot 10^6$ daltons) is cleaved to give two

---

Fig. 2. The circular restriction map of pBR313. (a) The relative position of restriction sites is drawn to scale on a circular map divided into units of $1 \cdot 10^5$ daltons. The restriction sites for *Alu*I, *Eco*RII, *Hae*II and *Hae*III represent only those which could be mapped. (b) The relationship between pMB9 and its $Ap^r$ derivatives before (pBR312) and after (pBR313, pBR315 and pBR316) *Eco*RI* digestion are represented linearly with respect to those restriction sites located in the $Tc^r$ and $Ap^r$ genes. The origin of replication in this plasmid has been localized by restriction endonuclease analysis and electron microscopic determinations (unpublished observations).

new fragments of 1.05 and 0.05 $\cdot 10^6$ daltons. Slots 2 and 3 show that *Sal*I and *Bam*HI digestions cleave the same 0.53 $\cdot 10^6$ dalton *Eco*RII fragment generating 0.29 and 0.24 $\cdot 10^6$ dalton fragments in the *Sal*I digest and 0.4 and 0.13 $\cdot 10^6$ daltons in case of the *Bam*HI digest. Since *Hin*dIII is shown to cleave one *Eco*RII fragment while both *Sal*I and *Bam*HI cleave a different fragment and the fact that *Hin*dIII and *Bam*HI sites are adjacent, indicated



that there is one *Eco*RII site between *Hin*dIII and *Bam*HI sites and no *Eco*RII site between *Bam*HI and *Sal*I sites. Combination digests with additional restriction endonucleases have enabled us to localize other *Eco*RII sites on pBR313 (Fig. 2a).

*(e) Mapping the substrate sites of the restriction endonucleases HincII, HpaI, SmaI, BglI, AluI, HaeII and HaeIII*

The restriction enzyme *Hin*cII recognizes the sequence

$$\begin{array}{l} G\ T\ Py\downarrow Pu\ A\ C \\ C\ A\ Pu\uparrow Py\ T\ G \end{array} \qquad \text{(Landy et al., 1974)}$$

As shown in Fig. 2a, there are four *Hin*cII sites in pBR313, one of which, is present in the $0.4 \cdot 10^6$ dalton *Pst*I fragment missing in pBR317. It should be noted at this point that the *Hpa*I-*Hin*cII and *Sal*I-*Hin*cII double digestion patterns are identical to the *Hin*cII pattern (data not shown). This is due to the purine-pyrimidine ambiguity present in the *Hin*cII substrate site which enables this particular enzyme to recognize both the *Hpa*I

$$\begin{array}{l} G\ T\ T\downarrow A\ A\ C \\ C\ A\ A\uparrow T\ T\ G \end{array} \quad \text{and } \textit{Sal}\text{I} \quad \begin{array}{l} G\downarrow T\ C\ G\ A\ C \\ C\ A\ G\ C\ T\uparrow G \end{array} \quad \text{substrate}$$

sites (Danna et al., 1973; Bolivar and Shine, 1976, unpublished observation).

As shown in Fig. 2a, the *Eco*RI site is located $0.29 \cdot 10^6$ daltons from the *Hin*cII situated in the $Ap^r$ gene (S. Falkow, personal communication) and $0.35 \cdot 10^6$ daltons from the *Hin*cII-*Sal*I site. Since a *Sal*I-*Hpa*I double digestion generates a $1.3 \cdot 10^6$ dalton fragment which is also present in a *Hin*cII digest, this places the *Hpa*I site clockwise of the *Sal*I site as shown in Fig. 2a. When the $2.29 \cdot 10^6$ dalton fragment produced by a *Hin*cII digest of pBR313 is cleaved by *Sma*I, this fragment is reduced by $0.12 \cdot 10^6$ daltons. The fact that

---

Plate II. (a) Analysis of *Pst*I and *Pst*I-*Eco*RI single and double digestions of pMB9, pRSF2124, pBR312 and pBR313 using agarose gel electrophoresis. Molecular weights estimates are based on the 6 λ fragments generated by *Eco*RI, the linear forms of the plasmids pMB8 and pVH51 and the *Hin*dIII generated fragments of the SV40 genome (slots 1, 6 and 11). The *Pst*I digestion patterns of the various plasmids are as follows: (slot 2) pMB9; (slot 3) pSF2124; (slot 4) pBR312 and (slot 5) pBR313. *Pst*I-*Eco*RI double digestions of these plasmids are as follows: (slot 7) pMB9; (slot 8) pSF2124; (slot 9) pBR312 and (slot 10) pBR313.
(b) Acrylamide slab gel electrophoresis of *Eco*RII cleaved pMB9, pBR312, pBR313 and pBR316 DNAs. Purified plasmids DNA were cleaved with *Eco*RII as described in MATERIALS AND METHODS and the fragments were dialyzed and subjected to acrylamide gel electrophoresis. The seven PM2 *Hin*dIII-generated fragments were used as molecular weight markers (slots 4, 7 and 10). Slots 5, 6, 8 and 9 show the *Eco*RII pattern of plasmids pBR313, pBR316, pBR312 and pMB9 respectively. Double digestions *Eco*RII-*Hin*dIII, *Eco*RII-*Sal*I and *Eco*RII-*Bam*HI of pBR313 DNA are shown in slots 1, 2 and 3 respectively. For explanation see the text (RESULTS Section (d)).

the $1.42 \cdot 10^6$ dalton fragment produced by a *Sal*I-*Sma*I double digest is also reduced by $0.12 \cdot 10^6$ daltons when digested with *Hpa*I, places the *Sma*I site $0.12 \cdot 10^6$ daltons clockwise from the *Hpa*I site (Fig. 2a).

Combination digest of pBR313 and derivative pBR plasmids (Bolivar et al., 1977) have enabled us to map the five *Bgl*I restriction sites shown in Fig. 2a.

The *Alu*I restriction enzyme cleaves pBR313 into more than eighteen fragments, some of which have been mapped by analysis of double digestion patterns. At present, we have been able to localize seven *Alu*I sites on pBR313. The *Alu*I site between the *Eco*RI and *Hin*dIII sites (Fig. 2a) was localized by the determination of the nucleotide sequence in this region of the DNA (J. Shine, unpublished observation). Using the strategy of combination digests, we were also able to map eleven *Hae*II sites and 4 *Hae*III sites present in pBR313 (Fig. 2a).

*(f) Molecular cloning of various restriction endonuclease-generated fragments in pBR313*

DNA fragments from various sources were produced by digestion with *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*I and *Hin*dIII-*Bam*HI restriction enzymes and cloned in their respective sites in pBR313 (Table I). *Eco*RI-recombinant plasmids of pBR313 gave $Ap^r$-$Tc^r$ phenotypes while *Bam*HI, *Sal*I and *Hin*dIII-*Bam*HI-recombinant plasmids were $Ap^r$-$Tc^s$. While some transformant-carrying *Hin*dIII-recombinant plasmids were $Ap^r Tc^s$, others were found to have a low-level $Tc^r$ which was observed when recombinant transformants were incubated for more than 24 h on Luria agar plates containing 10 μg/ml Tc. As in the case of the *Eco*RI recombinant plasmid, cloning of DNA fragments into the *Hpa*I or *Sma*I sites of pBR313 does not affect the expression of $Tc^r$ (data not shown).

*(g) Tetracycline-cycloserine enrichment for recombinant transformants*

Since transformation of *E. coli* K12 with in vitro ligated recombinant DNA may yield recombinant transformants at a frequency as low as $10^{-6}$ to $10^{-7}$/ml/μg of DNA, a procedure was needed for enriching the number of recombinant transformants in the total cell population. Such a technique was developed by taking advantage of the bacteriostatic nature of Tc and the bactericidal effect of Ap and Cyc. The rationale behind this procedure is the temporary inhibition of the growth of $Tc^s$-recombinant transformants by the addition of Tc to the growth medium. After allowing the $Tc^r$-transformants a 45 min interval of exposure to Tc, Cyc was added at a concentration which promoted the exponential lysis of growing cells (Curtiss et al., 1965). The $Tc^s$-recombinant cells can be recovered after the removal of the Tc and Cyc. Nontransformed cells can be eliminated from the culture by the addition of Ap either before or after the Tc-Cyc lytic step. A mixed-culture reconstruction experiment was conducted to demonstrate the practicality of this rationale. As shown in Table II, recombinant transformants containing *N. crassa* DNA (pBR313-NCS8) initially present at a frequency of $1 \cdot 10^{-6}$ were enriched to $3 \cdot 10^{-1}$ by this procedure.

TABLE I

MOLECULAR CLONING OF VARIOUS DNA FRAGMENTS IN pBR313

The molecular weights ($M_r \cdot 10^{-6}$) under each restriction site represent DNA fragments of independent clones isolated in this laboratory.

| DNA sources | Restriction endonuclease substrate site | | | | |
|---|---|---|---|---|---|
| | *Eco*RI | *Hin*dIII | *Bam*HI | *Sal*I | *Hin*dIII-*Bam*HI |
| *E. coli* | | | | | |
| *Eco*RI modification methylase | | 1.0 | | | |
| Chloramphenicol resistance[a] | | | | | 3.65 |
| *D. melanogaster* | | 3.1 | 5.8 | 2.0 | |
| | | 4.0 | 4.82 | | |
| | | 0.5 | 4.5 | | |
| | | 1.2 | 2.6 | | |
| | | | 0.86 | | |
| *N. crassa* | 2.5 | 1.05 | 3.5 | 3.2 | |
| | | 0.8 | 1.5 | 3.05 | |
| | | 0.5 | 8.0 | 1.8 | |
| | | 0.3 | 4.0 | | |
| | | 3.1 | 0.5 | | |
| | | 4.2 | | | |
| Cauliflower | | | 4.8 | | |
| Mosaic virus | | | 3.2 | | |
| (Cabbage B strain)[b] | | | 1.6 | | |

[a] Derived from an uncharacterized R-factor originally contained in a strain of *S. typhimurium* (unpublished observations).
[b] Meagher et al.

An example of the utility of pBR313 as a cloning vector in conjunction with this enrichment technique can be illustrated by the following results. *Drosophila melanogaster* DNA and pBR313 were cleaved separately with *Bam*HI and *Sal*I restriction endonucleases. After in vitro ligation, the DNA was used to transform RR1. When the transformed culture reached saturation, the percentages of $Ap^r$ cells in cultures from the *Sal*I and *Bam*HI experiments were 0.023% and 0.16% respectively. Samples of the transformed culture were diluted 1/50 and after logarithmic growth was established, Ap was added. The stationary phase cultures were found to be approximately 100% $Ap^r$. The enriched cultures were diluted 1/100 (one liter total) and incubated for 1 h before Tc was added. After 45 min incubation in the presence of Tc, Cyc was added to the cultures and incubated for 2.5 h. The

TABLE II

RECONSTRUCTION EXPERIMENT DEMONSTRATING THE TETRACYCLINE-CYCLOSERINE ENRICHMENT FOR RECOMBINANT TRANSFORMANTS

Tetracycline-cycloserine enrichment for recombinant plasmids of pBR313.
Tetracycline (Sigma) was added to transformed cultures in logarithmic growth phase to a final concentration of 10 μg/ml. After a period of 45 min, D-cycloserine (Cyc) (Sigma) was added to a final concentration of 102 μg/ml for a period of 60 min. Surviving cells were harvested by centrifugation, washed once and resuspended in Luria broth. For eliminating non-transformants from the culture, ampicillin (Ayerst Laboratories) was added to a final concentration of 20 μg/ml for 4 to 12 h either before or after the Tc-Cyc lytic step.

| Strains | Drug susceptibility | Relative frequency of input cells before treatment | | Step 1 | Step 2 | Step 3 | Relative frequency of input cells after treatment |
|---|---|---|---|---|---|---|---|
| RR1 | $Ap^S$ $Tc^S$ | RR1 | $9.9 \cdot 10^{-1}$ | Add ampicillin to a final conc. of 20 μg/ml for 4 to 12 h at 37°C | Remove ampicillin; add tetracycline to a final conc. of 10 μg/ml for 1 h at 37°C | Add D-cycloserine to a final conc. of 100 μg/ml for 1 h at 37°C; wash cells, grow overnight at 37°C | $6.9 \cdot 10^{-1}$ |
| RR1 (pBR313) | $Ap^r$ $Tc^r$ | pBR313 | $1 \cdot 10^{-5}$ | | | | $3 \cdot 10^{-5}$ |
| RR1 (pBR313-NCS8)[a] | $Ap^r$ $Tc^S$ | pBR313-NCS8 | $1 \cdot 10^{-6}$ | | | | $3 \cdot 10^{-1}$ |

[a] pBR313 contains *Sal*I digested *Neurospora crassa* DNA cloned in the *Sal*I site of the $Tc^r$ gene.

cultures were centrifuged and resuspended in 100 ml of growth medium without antibiotics and incubated for 10 h. The percentages of cells that were $Ap^rTc^s$ (i.e., cells carrying recombinant plasmids) in the *Sal*I and *Bam*HI experiments were enriched to 92% and 88% respectively.

## DISCUSSION

By means of the *Eco*RI and *Eco*RI* reactions and the genetic translocation of ampicillin resistance translocon (TnA), we have constructed a series of bacterial plasmid cloning vectors with a ColE1 replication mode. These plasmids have been characterized with thirteen restriction enzymes. One of these plasmids, pBR313, has some features which make it a more efficient cloning vehicle than the currently used vectors, for example pSC101 (Cohen et al., 1973), ColE1 (Hershfield et al., 1974), pMB9, and phage lambda (Cameron et al., 1975). The advantages of using pBR313 as a cloning vector are summarized as follows. (1) The molecular cloning of *Eco*RI, *Hin*dIII, *Bam*HI, *Sal*I, *Hpa*I and *Sma*I can now be achieved in a single low molecular weight, amplifiable plasmid. (2) The substrate sequences for *Hin*dIII, *Bam*HI and *Sal*I restriction endonuclease are located in $Tc^r$ region thus facilitating the recovery of cells harboring recombinant DNA by virtue of their $Ap^r$-$Tc^s$ phenotypes. Although recombinant plasmids generated by *Eco*RI, *Hpa*I and *Sma*I do not inactivate the $Tc^r$ mechanism in pBR313, double-digested DNA fragments involving any one of these enzymes and either *Hin*dIII, *Bam*HI or *Sal*I will produce $Tc^s$ recombinants. We believe that the use of these six restriction enzymes and the 14 possible combination digests will provide not only the opportunity for cloning many interesting DNA fragments but also the further dissection of these DNAs into their component parts. This feature is of particular importance for the DNA sequencing technique recently developed by Maxam and Gilbert (Maxam and Gilbert, 1977). (3) Recombinant transformants with $Ap^r$-$Tc^s$ phenotypes are amenable to enrichment over non-recombinant and non-transformant cells by the use of the Ap-Tc-Cyc lytic procedure. (4) As a result of the *Eco*RI* digestion of pBR312, the $Ap^r$ gene can no longer be translocated from pBR313 (S. Falkow, personal communication). This eliminates the possibility of translocating cloned DNA from the plasmid vector to either the chromosome or other resident episomes.

While cloning into the *Bam*HI and *Sal*I sites in pBR313 clearly inactivates the $Tc^r$ mechanism, cloning in the *Eco*RI site does not. Many DNA fragments inserted into the *Hin*dIII site also inactivate the $Tc^r$ mechanism; however, other pieces of DNA only reduce the level of Tc resistance. Preliminary data suggest that the *Hin*dIII site may be localized in a regulatory region, i.e., a promoter for *E. coli* DNA polymerase. This notion is supported by the fact that the *Hin*dIII site in pBR313 is protected from digestion in the presence of RNA polymerase (Rodriguez et al., 1977).

At present, we have mapped more than forty restriction sites in pBR313 using thirteen restriction endonucleases. At least fourteen of these sites are

located in the $Tc^r$ gene complex, and we are in the process of mapping additional substrate sites in this region. This mapping will enable us to sequence the $Tc^r$ gene(s) according to the procedure of Maxam and Gilbert (Maxam and Gilbert, 1977) which we hope will provide new insight into the $Tc^r$ mechanism and its mode of control. Furthermore, our knowledge of these restriction sites in pBR313 has enabled us to construct other cloning vectors which permit the use of *Pst*I and *Hinc*II restriction enzymes for the molecular cloning of DNA into the $Ap^r$ gene (Fig. 2a) (Bolivar et al., 1977). Since *Hinc*II is known to produce blunt-ended cleavages in DNA, a cloning vector with one *Hinc*II site either in the Ap or Tc genes would allow for the cloning of any blunt-ended DNA fragment by ligation with phage T4 polynucleotide DNA ligase under the appropriate conditions (Sgaramella et al., 1970; Heyneker et al., 1976).

ACKNOWLEDGEMENTS

This work was supported by grants to H.W.B. from the National Science Foundation and the National Institutes of Health. R.L.R. was supported by a postdoctoral fellowship from the A.P. Giannini Foundation for Medical Research. We would also like to acknowledge Dr. Istvan Fodor and Alejandra Covarrubias for their discussion and technical assistance. We are also grateful to Patricia L. Clausen for her expert preparation of this manuscript.

REFERENCES

Betlach, M.C., Hershfield, V., Chow, L., Brown, W., Goodman, H.M. and Boyer, H.W., Fed. Proc. 35 (1976) 2037—2043.
Bolivar, F., Rodriguez, R.L., Greene, P., Betlach, M.C., Boyer, H.W., Crosa, J. and Falkow, S., Gene, 2 (1977) 000—000.
Boyer, H.W. and Roulland-Dussoix, D., J. Mol. Biol., 41 (1969) 459—472.
Cameron, J.R., Panasenko, S.M., Lehman, R.R. and David, R., Proc. Natl. Acad. Sci. USA, 72 (1975) 3416—3420.
Clewell, D.B., J. Bacteriol., 110 (1972) 667—676.
Clewell, B.B. and Helinski, D., Proc. Natl. Acad. Sci. USA, 62 (1969) 1159—1166.
Cohen, S.N., Chang, A.C.Y. and Hsu, C.L., Proc. Natl. Acad. Sci. USA, 69 (1972) 2110—2114.
Cohen, S.N., Chang, A.C.Y., Boyer, H.W. and Helling, R., Proc. Natl. Acad. Sci. USA, 70 (1973) 3240—3244.
Curtiss, R. III, Charamella, L.J., Berg, C.M. and Harris, P., J. Bacteriol., 91 (1965) 1238—1251.
Danna, K.J., Sack, G.H. and Nathans, D., J. Mol. Biol., 78 (1973) 363—376.
Datta, N. and Richmond, M.D., Biochem. J., 98 (1966) 204.
Greene, P.J., Betlach, M.C., Goodman, H.M. and Boyer, H.W., in R.B. Wickner (Ed.), Methods in Molecular Biology Series; DNA Replication and Biosynthesis, vol. 7, Marcel Dekker, New York, 1974, pp. 87—111.
Gromkova, R. and Goodgal, S.H., J. Bacteriol., 109 (1972) 987.
Guerry, P., LeBlanc, D.J. and Falkow, S., J. Bacteriol., 116 (1973) 1064—1066.
Hamer, D. and Thomas, C., Proc. Natl. Acad. Sci. USA, 78 (1976) 1537—1541.
Heffron, F., Rubens, C. and Falkow, S., Proc. Natl. Acad. Sci. USA, 72 (1975) 3623—3527.

Heffron, R., Bedinger, P., Champoux, J.J. and Falkow, S., Proc. Natl. Acad. Sci. USA, 74 (1977) 702—706.
Hershfield, V., Boyer, H.W., Lovett, M., Yanofsky, C. and Helinski, D., Proc. Natl. Acad. Sci. USA, 71 (1974) 3455—3461.
Hershfield, V., Boyer, H.W., Chow, L. and Helinski, D., J. Bacteriol., 126 (1976) 447—453.
Heyneker, H.L., Shine, J., Goodman, H.M., Boyer, H.W., Rosenberg, Dickerson, J., Narang, S.A., Itakura, K., Lin, S. and Riggs, A.D., Nature, 263 (1976) 748—752.
Heyneker, H., Greene, P.J., Betlach, M.C., Bolivar, F., Rodriguez, R., Covarrubias, A., Fodor, I. and Boyer, H.W., (1977) Manuscript in preparation.
Landy, A., Ruedisueli, E., Robinson, L., Foeller, C. and Ross, W., Biochemistry, 13 (1974) 2134—2141.
Levy, S. and McMurray, L., Biochem. Biophys. Res. Commun., 56 (1974) 1060—1068.
Maxam, A.M. and Gilbert, W., Proc. Natl. Acad. Sci. USA, 74 (1977) 560—564.
Meagher, R.B., Tait, R.C., Betlach, M. and Boyer, H.W., Cell, 10 (1977) 521—536.
Nagel De Zwaig, R. and Luria, S., J. Bacteriol., 94 (1967) 1112—1119.
Padmanabhan, R. and Wu, R., J. Mol. Biol., 65 (1972) 447—464.
Panet, A., Van de Sande, J.H., Loewen, P.C., Khorana, H.G., Raae, A.J., Lillehaug, J.R. and Kleppe, K., Biochemistry, 12 (1973) 5045—5050.
Polisky, B., Greene, P.J., Garfin, D.E., McCarthy, B.J., Goodman, H.M. and Boyer, H.W., Proc. Natl. Acad. Sci. USA, 72 (1975) 3310—3314.
Roberts, R.J., Breitmeyer, J.B., Tabachuik, N.F. and Myers, P.A., J. Mol. Biol., 91 (1974) 121—123.
Roberts, R.J., Myers, P.A., Morrison, A. and Murray, K., J. Mol. Biol., 102 (1976) 157—165.
Rodriguez, R.L., Bolivar, F., Goodman, H.M., Boyer, H.W. and Betlach, M.C., in D.P. Nierlich, W.J. Rutter and C.F. Fox (Eds.), Molecular Mechanisms in the Control of Gene Expression (ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. V), Academic Press, New York, 1976, pp. 471—477.
Rodriguez, R.L., Tait, R., Shine, J., Bolivar, F., Heyneker, H.L., Betlach, M. and Boyer, H.W., Characterization of tetracycline and ampicillin resistant plasmid cloning vehicles, in Molecular Cloning of Recombinant DNA, Vol. 13 (Ninth Miami Winter Symposia), 1977, in press.
Schwartz, S.A. and Helinski, D., J. Biol. Chem., 216 (1971) 6318—6327.
Sgaramella, V., Van de Sande, J.H. and Khorana, H.G., Proc. Natl. Acad. Sci. USA, 67 (1970) 1468—1475.
So, M., Gill, R. and Falkow, S., Mol. Gen. Genet., 142 (1976) 239—249.
Tait, R.C., Rodriguez, R.L. and Boyer, H.W., Mol. Gen. Genet., 151 (1977) 327—331.
Wilson, G.A. and Young, F.E., J. Mol. Biol., 97 (1975) 123—126.
Yoshimori, R., Roulland-Dussoix, D. and Boyer, H.W., J. Bacteriol., 112 (1972) 1275—1283.

Communicated by D.R. Helinski.