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# CONSTRUCTION AND CHARACTERIZATION OF NEW CLONING VEHICLES
## II. A MULTIPURPOSE CLONING SYSTEM

(Recombinant DNA; molecular cloning plasmid vector; EK2 host-vector system)

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SUMMARY

In vitro recombination techniques were used to construct a new cloning vehicle, pBR322. This plasmid, derived from pBR313, is a relaxed replicating plasmid, does not produce and is sensitive to colicin E1, and carries resistance genes to the antibiotics ampicillin (Ap) and tetracycline (Tc). The antibiotic-resistant genes on pBR322 are not transposable. The vector pBR322 was constructed in order to have a plasmid with a single *Pst*I site, located in the ampicillin-resistant gene ($Ap^r$), in addition to four unique restriction sites, *Eco*RI, *Hin*dIII, *Bam*HI and *Sal*I. Survival of *Escherichia coli* strain X1776 containing pBR313 and pBR322 as a function of thymine and diaminopimelic acid (DAP) starvation and sensitivity to bile salts was found to be equivalent to the non-plasmid containing strain. Conjugal transfer of these plasmids in bi- and triparental matings were significantly reduced or undetectable relative to the plasmid ColE1.

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Abbreviations: $Ap^r$, ampicillin-resistant; $Cm^r$, chloramphenicol-resistant; $Col^{imm}$, colicin immunity; DAP, diaminopimelic acid; DTT, dithiothreitol; $Km^r$, kanamycin-resistant; LB, Luria broth; $Nx^r$, nalidixic-resistant; SDS, sodium dodecyl sulfate; $Sm^r$, streptomycin-resistant; $Su^r$, sulfonamide-resistant; $Tc^r$, tetracycline-resistant.

## INTRODUCTION

Bacterial plasmids and bacteriophage have a key role in recombinant DNA technology. Segments of DNA from diverse origins can be excised with the appropriate restriction endonuclease and added to plasmids or bacteriophage (Hershfield et al., 1974; Morrow et al., 1974; Cameron et al., 1975). If these new molecules contain an intact replicon, they can be propagated in a suitable host to yield large quantities of recombinant DNA and in some instances, specific gene products (Hershfield et al., 1974). Several bacterial plasmids have been used as cloning vectors: pSC101 (Cohen et al., 1973), ColE1 (Hershfield et al., 1974) and pCR1 (Covey et al., 1976). However, these plasmids and their derivatives (Hamer et al., 1975; Hershfield et al., 1976; So et al., 1976) have limited versatility in terms of genetic markers for selection of transformants and screening for recombinant plasmids.

We have described the construction of a series of plasmids containing Ap- and Tc-resistant genes derived from pRSF2124 (So et al., 1976) and pSC101 respectively in combination with replication elements of a ColE1-like plasmid (Betlach et al., 1976; Rodriguez et al., 1976). One of these plasmids, pBR313, provides single cleavage sites for the *Hin*dIII, *Bam*HI, *Eco*RI, *Hpa*I, *Sal*I and *Sma*I restriction endonucleases (Bolivar et al., 1977). In the case of the *Hin*dIII, *Bam*HI and *Sal*I endonuclease cloning sites, the insertion of DNA fragments inactivates the $Tc^r$ gene. In this paper, we report the construction of another plasmid (pBR322) which is less than half the size of pBR313 and provides additional cloning advantages. The plasmid pBR322 contains a unique *Pst*I cleavage site located in the $Ap^r$ gene as well as two *Hin*cII sites located in the $Ap^r$ and $Tc^r$ genes. The *Pst*I site can be used for molecular cloning of DNA fragments via homodeoxy polymeric extension (Lobban and Kaiser, 1973) and the *Hin*cII site for blunt-end ligation techniques (Sgaramella et al., 1970; Sugino et al., 1977). The properties of pBR313 and pBR322 in the *E. coli* strain X1776 are also presented.

## MATERIAL AND METHODS

### (a) Bacterial strains

*E. coli* K12 strain RR1 $F^-$ *pro leu thi lac*Y $Str^r$ $r_K^-$ $m_K^-$ was used as the recipient cells in the transformation experiments. *E. coli* B strain HB50 *pro leu try his arg met thr gal lac*Y $Str^r$ was used to prepare unmethylated plasmid DNA for *Eco*RII digestions (Yoshimori et al., 1972). *E. coli* K12 strain X1776 $F^-$ *tau*A53 *dap*D8 *mer*A1 *sup*E42 Δ40(*gal-uvr*B) $\lambda^-$ *min*B2 *mal*A25 *thy*A57 *met*C65 Δ29(*bio*H-*asd*) *cys*B2 *cyc*A1 *Hsd*R2 was kindly provided by R. Curtiss III.

### (b) Media and buffers

For transformation RR1 was grown in either LB or M9-glucose minimal media, before $CaCl_2$ treatment. X1776 was also grown in LB supplemented

with DAP 200 μg/ml and thymine (thy) 50 μg/ml. The BSG buffer solution used for washing X1776 in the DAP-less death experiments was 0.85% NaCl, 0.03% $KH_2PO_4$, 0.06% $Na_2HPO_4$ 100 μg/ml gelatin.

*(c) Preparation of plasmid DNA*

Plasmid DNA was prepared by first amplifying M9-glucose-grown cultures by the addition of 170 μg/ml of chloramphenicol during logarithmic phase of growth (Clewell et al., 1972). Extraction and purification of plasmid DNA was achieved by a cleared lysate technique previously described (Betlach et al., 1976).

*(d) Enzymes*

All the restriction enzymes used in this work, except for *Hpa*I (BRL laboratories) were purified according to the procedure by Greene et al. (1977) and are itemized in Table I. Reaction conditions for the various restriction endonucleases have been described previously (Bolivar et al., 1977). T4 DNA ligase was purified from T4 am N82 infected *E. coli* B, according to the procedure described by Panet et al. (1973). The final preparation (500 U/ml) was homogeneous as judged by SDS-polyacrylamide gel electrophoresis.

*(e) Ligation of DNA*

Ligations were carried out in 66 mM Tris—HCl pH 7.6, 6.6 mM $MgCl_2$ 10 mM DTT and 0.5 mM ATP at 12°C for 2—12 h. The concentration of T4 DNA ligase and of DNA termini varied to promote polymerization or circularization. When blunt-ended DNA fragments were ligated, the concentration of ends was at least 0.2 μM and approximately 50 U of T4 DNA ligase per ml was added to the reaction mixture (Heyneker et al., 1976). When DNA fragments with cohesive ends were ligated, 5 U of T4 DNA ligase per ml was sufficient and the concentration of ends was adjusted in such a way that linear molecules were favored (Dugaiczyk et al., 1975).

*(f) Agarose and acrylamide gel electrophoresis*

The conditions for agarose and acrylamide electrophoresis have been previously described (Bolivar et al., 1977).

*(g) Transformation of E. coli K12*

*E. coli* RRI cells were prepared for transformation by the method described by Cohen et al. (1972). 100 μl of DNA in 30 mM $CaCl_2$ were added to 200 μl of $CaCl_2$-treated cells ($5 \cdot 10^9$ cells/ml) and the mixture was chilled in ice for 60 min, after which it received a 75-sec, 42°C heat pulse. The pulse was terminated with the addition of 3 ml of LB. The cells were grown for 2 h at 37°C before plating. Transformation of X1776 was achieved using the procedure described by R. Curtiss III (personal communication). An overnight culture of X1776 in LB + DAP + thy was diluted 1/10 with 20 ml of fresh LB + DAP + thy and incubated in a shaker et 37°C for 3 to 4 h until the

TABLE I

RESTRICTION ENDONUCLEASES

| Endonucleases | Substrate site | Reference | Endonuclease | Substrate site | Reference |
|---|---|---|---|---|---|
| *Alu*I | A G↓C T | Roberts et al., 1976 | *Hae*III | G G↓C C | Roberts et al., unpublished observations |
| *Bam*HI | G↓G A T C C | Wilson and Young, 1975 | *Hinc*II | G T Py↓PuA C | Landy et al., 1974 |
| *Bgl*I | --- | Wilson and Young, unpublished observations | *Hind*III | A↓A G C T T | Danna et al., 1973 |
| *Eco*RI | G↓A A T T C | Greene et al., 1976 | *Hpa*I | G T T↓A A C | Gromkova and Goodgal, 1972 |
| *Eco*RII | ↓C C $^{A}_{T}$ G G | Yoshimori et al., 1975 | *Pst*I | C T G C A↓G | Smith et al., 1976 |
| *Hae*II | PuG C G C↓Py | Roberts et al., unpublished observations | *Sal*I | G↓T C G A C | Bolivar et al., 1977 |

culture reached an absorbance of 0.5 to 0.6 $A_{600}$. The culture was centrifuged at 7000 rpm for 10 min at 4°C, and the cells washed in 10 ml cold 10 mM NaCl. The suspension was again centrifuged as above and the pellet resuspended in 10 ml of freshly prepared cold 75 mM $CaCl_2$ (pH 8.4) and placed in ice for 25 min. Cells were centrifuged as described above and the pellet resuspended in 2 ml of 75 mM $CaCl_2$ pH 8.4, of which 200 μl was added to 100 μl of plasmid DNA in 10 mM Tris pH 8. The mixture was kept in ice for 60 min, then heated 60 sec at 42°C. Tubes were chilled for 10 min and 3 ml of LB + DAP + thy were added. The cells were incubated at 37°C for 3 h and plated in selective media. The plates were incubated 2 to 3 days at 37°C.

## RESULTS

### *I. Construction of pBR321 and pBR322*

We have described the construction of a series of cloning vehicles, one of which, pBR313, a 5.8 · $10^6$ dalton $Ap^r$ $Tc^r$ $Col^{imm}$ plasmid (Fig.1), has been extensively mapped using 14 restriction endonucleases (Bolivar et al., 1977). Experiments with pBR313 indicated that one of its *Pst*I sites was located in the $Ap^r$ gene. Therefore molecular cloning into this *Pst*I site would result in recombinant molecules which could be detected by screening for $Ap^s$ phenotypes. In order to construct a molecular cloning vector with one *Pst*I site in the $Ap^r$ gene, it was necessary to construct two derivatives of pBR313. An $Ap^sTc^rCol^{imm}$ plasmid, pBR318, containing one *Pst*I site was obtained by transforming *E. coli* RRI with ligated *Pst*I fragments of pBR313 and selecting for $Tc^r$ transformants. $Tc^r$ transformants which were $Ap^s$ were found to carry plasmids that lack the 1.25 and 0.42 · $10^6$ dalton *Pst*I fragments present in pBR313 (Fig.1). Another pBR313 derivative, pBR320, an $Ap^rTc^sCol^s$ plasmid with a molecular weight of 1.95 · $10^6$ daltons was obtained by transforming *E. coli* RRI with unligated *Eco*RII fragments of pBR313 and selecting for $Ap^r$ transformants. Sixteen $Ap^rTc^s$ clones were examined, and one was found to carry a plasmid, pBR320, containing only one *Pst*I site. This clone was found to be sensitive to colicin E1. Fig. 1 shows a tentative restriction endonuclease map of pBR320.

An in vitro recombination experiment using pBR318 and pBR320 was designed to restore the $Ap^rTc^r$ markers in a single low molecular weight relaxed plasmid containing one *Pst*I substrate site. The construction of this plasmid was accomplished by the digestion of pBR318 with *Pst*I and *Hpa*I endonucleases which resulted in two pieces of DNA with molecular weights of 1.95 and 2.2 · $10^6$ daltons; the smaller DNA fragment carried the $Tc^r$ gene(s) (Rodriguez et al., 1976; Tait et al., 1976; Bolivar et al., 1977) and part of the $Ap^r$ gene as shown in Fig. 1. The plasmid pBR320 was cleaved with the restriction enzymes *Pst*I and *Hin*cII to yield three fragments of DNA. The largest fragment, 1.15 · $10^6$ daltons, carries the "origin" of replication and the remaining portion of the $Ap^r$ gene not present in the 1.95 · $10^6$ dalton fragment of pBR318.

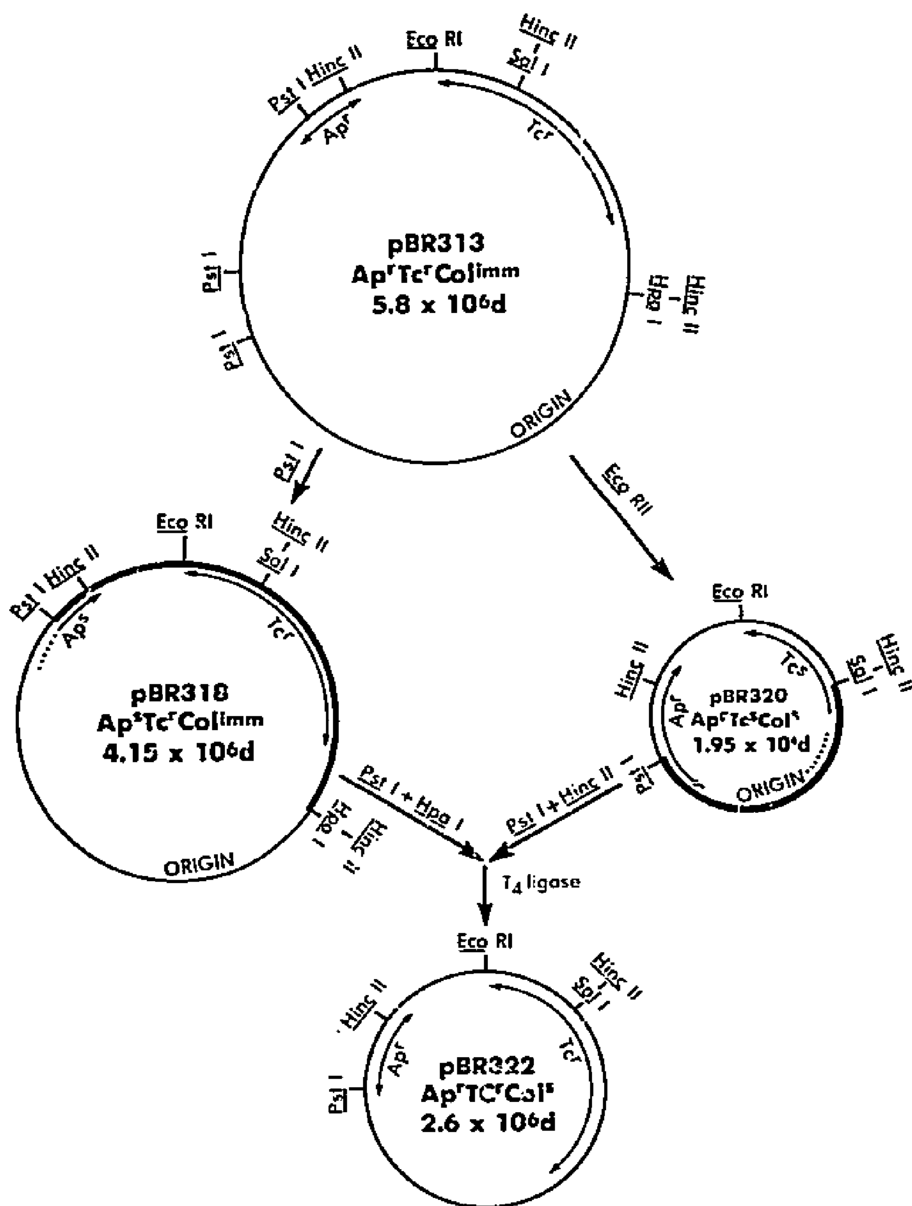


Fig. 1. Diagrammatic representation of the construction of pBR322. The parental plasmid pBR313 was used to construct pBR318 and pBR320 by *Pst*I and *Eco*RII endonuclease digestions respectively. These two plasmids were separately digested with *Pst*I and *Hpa*I endonucleases (pBR318) and with *Pst*I and *Hin*cII endonucleases (pBR320). The heavy lined regions from the *Pst*I to *Hpa*I sites in pBR318 and from *Pst*I to *Hin*cII sites (*Sal*I) in pBR320 represent the two DNA fragments that were ligated to each other to generate pBR321 and pBR322. The origins of replication in these plasmids were determined by restriction endonuclease analysis and electron microscopic examinations (unpublished observations). For detailed explanation, see the text.

The digested DNAs were mixed, ligated in vitro, and transformed into *E. coli* RRI. Since neither pBR320 nor the 1.95 · $10^6$ dalton fragment of pBR318 carry the colicin E1 immunity gene, transformants were selected for $Ap^rTc^r$ and then screened for sensitivity to colicin E1. This transformation yielded numerous $Ap^rTc^rCol^s$ clones which carried plasmids (e.g. pBR 321) with a molecular weight of 3.1 · $10^6$ daltons. As expected, this plasmid resulted from the addition of the 1.95 · $10^6$ dalton *HpaI-PstI* fragment of pBR318 and the 1.15 · $10^6$ d. *PstI-HincII* DNA piece from pBR320.

From this transformation we obtained in one instance a smaller, 2.6 · $10^6$ dalton, $Ap^rTc^rCol^s$ plasmid. This plasmid, possibly the result of in vivo recombination event near unligated termini, was missing 0.5 · $10^6$ daltons of DNA from a region of pBR321, not associated with $Ap^r$, $Tc^r$ or DNA replication (Fig. 2). Because of its lower molecular weight compared with pBR 321, pBR 322 was chosen for further characterization of number and position of restriction sites.

*II. Mapping of pBR322 restriction endonuclease digestions*

As determined by agarose and acrylamide gel electrophoresis of DNA digests, pBR322 was found to carry unique substrate sites for the *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I and *Sal*I restriction endonucleases. Double and triple endonuclease digests of the plasmid (data not shown) showed that the relative positions of these sites were identical to those mapped in pBR313. As can be seen in Figs. 2 and 3, there are only two *Hin*cII sites in pBR322, one located 0.17 · $10^6$ daltons from the *Pst*I site in the $Ap^r$ gene and the other in $Tc^r$ gene, which is also a *Sal*I site (Bolivar et al., 1977).

The *Eco*RII restriction endonuclease was used to further characterize pBR322. As shown in Fig. 4a (slot 3), pBR322 has five *Eco*RII sites which yield fragments of 1.25, 0.64, 0.53, 0.22 and 0.04 · $10^6$ daltons upon digestion. Slots 3, 4, 5, 6 and 7 (Fig. 4a) show respectively, *Eco*RI, *Hinc*II, *Sal*I, *Bam*HI and *Bgl*I digestions of *Eco*RII-digested pBR322 DNA. In the case of the *Eco*RI endonuclease digest, the largest *Eco*RII fragment when cleaved gives two new fragments of 0.08 and 1.07 · $10^6$ daltons. Slots 5 and 6 show that *Sal*I and *Bam*HI endonucleases cleave the same 0.53 · $10^6$ dalton *Eco*RII fragment as in pBR313 (Bolivar et al., 1977) and generates 0.29 and 0.24 · $10^6$ dalton DNA fragments after the *Sal*I endonuclease digestion and 0.4 and 0.13 · $10^6$ dalton fragments after *Bam*HI endonuclease digestion. Slot 4 shows that the same 0.53 · $10^6$ dalton *Eco*RII piece is cleaved by *Hin*cII endonuclease, generating the same 0.29 and 0.24 · $10^6$ dalton fragments that the *Sal*I endonuclease produces. The 1.25 · $10^6$ dalton *Eco*RII fragment is also cleaved by *Hin*cII endonuclease into 0.82 and 0.43 · $10^6$ dalton fragments. Slot 7 shows the double digestion pattern of pBR322 DNA using *Eco*RII and *Bgl*I endonucleases. Three *Eco*RII fragments are cleaved by *Bgl*I endonuclease into smaller pieces. The largest *Eco*RII fragment (1.25 · $10^6$ daltons) is cleaved into 0.57 and 0.67 · $10^6$ dalton fragments. *Bgl*I endonuclease also cleaves the 0.53 · $10^6$ dalton fragments into 0.47 and 0.06 · $10^6$ dalton

Fig. 2. *Hin*cII-*Eco*RI endonuclease analyses of pBR321 and pBR322. Molecular weight estimates are based in the seven PM2 fragments generated by the *Hin*dIII endonuclease (31.4, 1.34, 0.6, 0.31, 0.29, 0.14, and 0.06, the last one not seen in the gel, Wes Brown, personal communication) (slot 1). The *Hin*cII endonuclease and *Hin*cII-*Eco*RI endonuclease single and double digestions of pBR322 are shown in slots 3 and 2 respectively while the *Hin*cII endonuclease and *Hin*cII-*Eco*RI endonuclease digestions of pBR321 are shown in slots 5 and 4 respectively. It can be seen that the $0.64 \cdot 10^6$ *Hin*cII band present in pBR322 (slot 3) (see also Fig. 3) is also present in pBR321 (slot 5). This band carries the *Eco*RI site (slots 2 and 4). These data indicate that the spontaneous deletion that generates pBR322 does not extend to the $Ap^r$ or $Tc^r$ genes nor the region located in the small *Hin*cII fragment ($0.64 \cdot 10^6$ daltons) in pBR321.

Fig. 3. The circular restriction map of pBR322. The relative position of restriction sites are drawn to scale on a circular map divided into units of $1 \cdot 10^5$ daltons (outer circle) and 0.1 kilobases (inner circle). The estimated size of the $Ap^r$ and $Tc^r$ genes represented in the figure were determined indirectly on the basis of the reported values for the size of the TEM β-lactamase (Datta and Richmond, 1966) and the $Tc^r$-associated proteins detected in the minicell system (Levy and McMurry, 1974; Tait et al., 1976). 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 $Tc^r$ mechanism. The position and size of the $Tc^r$ region is also consistent with the orientation of the TnA in pBR26 (Bolivar et al., 1977). Only ten out of twelve *Hae*II and *Alu*I and seven out of seventeen *Hae*III substrate sites are represented on the circular map of pBR322. The position of two of the ten *Alu*I sites located at $1.8 \cdot 10^6$ daltons and $1.86 \cdot 10^6$ daltons were mapped on a $0.7 \cdot 10^6$ dalton plasmid which encompasses this region of pBR322 (unpublished observation).

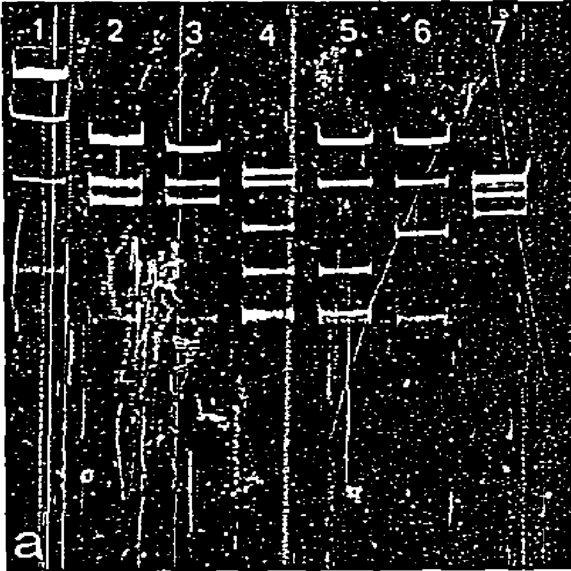


Fig. 4a. Acrylamide gel electrophoresis of *Eco*RII-cleaved pBR322 DNA. Analysis of *Eco*RII endonuclease (slot 2) and double endonuclease *Eco*RII-*Eco*RI digestions (slot 3), *Eco*RII-*Hin*cII (slot 4), *Eco*RII-*Sal*I (slot 5), *Eco*RII-*Bam*HI (slot 6) and *Eco*RII-*Bgl*I (slot 7) of pBR322 DNA. The seven *Hin*dIII-generated fragments from the DNA of phage PM2 (slot 1) with molecular weights of 3.5, 1.34, 0.6, 0.31, 0.29, 0.15 and 0.06 (Wes Brown, personal communication) were used as molecular weight standards. For explanation see the text.

pieces. The $0.22 \cdot 10^6$ dalton *Eco*RII fragment is cleaved by *Bgl*I endonuclease into 0.16 and $0.06 \cdot 10^6$ dalton DNA fragments. These data allow us to localize four *Eco*RII sites in the pBR322 map (Fig. 3).

The *Alu*I endonuclease cleaves pBR322 into approximately 12 fragments (Fig. 4b, slot 2). Four *Alu*I sites were mapped by analysis of the molecular weights of DNA fragments generated by double endonuclease digestion (Fig. 3). By comparing the *Alu*I fragments of pBR318 and pBR320 (data not shown), we have tentatively localized four additional *Alu*I sites in the map of pBR322. The *Alu*I site located between the *Eco*RI and the *Hin*dIII sites (Fig. 3) was determined by DNA sequencing (J. Shine et al., unpublished observations).

Using the same strategy of the analysis of double digestion patterns we were able to locate 10 out of 12 *Hae*II sites and the 3 *Bgl*I sites present in the pBR322 map (Fig. 3) (data not shown).

*III. Cloning properties of pBR322*

*(a) Cloning in the Tc$^r$ gene.* It has been previously shown (Rodriguez et al., 1976; Bolivar et al., 1977) that the *Hin*dIII, *Bam*HI and *Sal*I sites are present

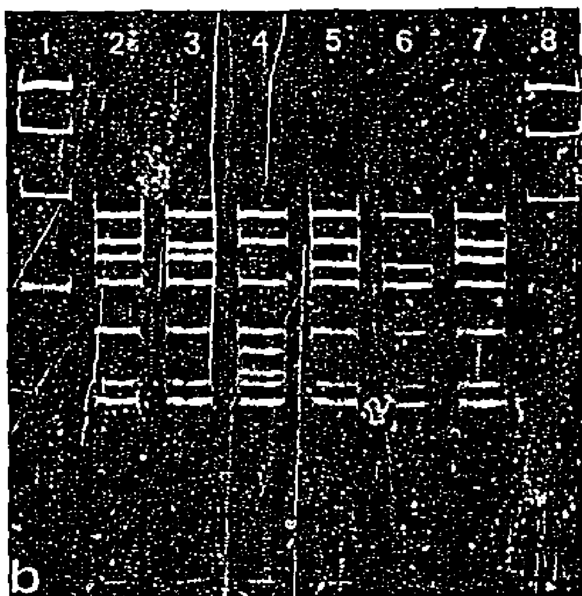


Fig. 4b. Acrylamide gel electrophoresis of *Alu*I endonuclease digested pBR322 DNA. Analysis of *Alu*I endonuclease (slot 2) and double digestions of *Alu*I-*Eco*RI (slot 3), *Alu*I-*Bam*HI (slot 4), *Alu*I-*Sal*I (slot 5), *Alu*I-*Hin*cII (slot 6), and *Alu*I-*Pst*I (slot 7) endonucleases of pBR322 DNA. The seven *Hin*dIII generated fragments from the DNA of phage PM2 (slot 1 and 8) were used as molecular weight standards. It can be seen that the second largest *Alu*I endonuclease generated fragment ($0.43 \cdot 10^6$ daltons) is cleaved by the *Eco*RI endonuclease generating a $0.42 \cdot 10^6$ daltons band and a $0.01 \cdot 10^6$ dalton fragment not seen in the gel. *Bam*HI endonuclease cleaves the $0.38 \cdot 10^6$ dalton fragment into two fragments of 0.2 and $0.18 \cdot 10^6$ daltons. *Sal*I endonuclease cleaves the same $0.38 \cdot 10^6$ dalton *Alu*I fragment generating a piece of $0.34 \cdot 10^6$ daltons and a small one of $0.04 \cdot 10^6$ daltons (not seen in the gel). *Hin*cII endonuclease cleaves two *Alu*I fragments; cleaves in the *Sal*I site generating the same $0.34 \cdot 10^6$ dalton *Alu*I fragment. The relative positions of these fragments can be seen in Fig. 3.

in the $Tc^r$ gene(s) carried by pBR313. Since these sites are in the same relative position in pBR322, we assumed they are associated with the $Tc^r$ gene(s) present in this plasmid. To confirm this point, DNA fragments from *E. coli*, *Drosophila melanogaster*, and *Neurospora crassa* were produced by digestion with *Hin*dIII, *Bam*HI and *Sal*I restriction endonucleases and cloned into their respective sites in pBR322. These recombinants were $Ap^rTc^s$ (Table II). Insertion of DNA fragments into the *Eco*RI site, as in pBR313, does not affect the expression of the $Tc^r$ mechanism.

*(b) Cloning in the $Ap^r$ gene.* 1. *Cloning into the PstI site.* To confirm the observation that the *Pst*I site in pBR322 is located in the $Ap^r$ gene, we have cloned several fragments of DNA in *Pst*I site of this plasmid (see Table II). *Pst*I generated fragments of the plasmid pMB1 (Betlach et al., 1976) were

ligated into the *Pst*I site of pBR322 and transformed into RR1. Transformants were selected for $Tc^r$ and screened for $Ap^s$ phenotypes. This experiment resulted in the cloning of five different *Pst*I fragments representing nearly the whole pMB1 genome (Table II).

The unique *Pst*I site in pBR322 provides two advantages for the molecular cloning of DNA by means of the homodeoxypolymeric DNA extention technique (Lobban and Kaiser, 1973). First, the *Pst*I site, which has the sequence C T G C A↓G (Smith et. al., 1976) provides a protruding 3′OH which

TABLE II

MOLECULAR CLONING OF VARIOUS DNA FRAGMENTS IN pBR322

| Sources | Restriction endonuclease substrate site | | | | | |
|---|---|---|---|---|---|---|
| | *Eco*RI | *Hin*dIII | *Bam*HI | *Sal*I | *Hin*cII[a] | *Pst*I |
| *N. crassa* | 2.8<br>1.1 | 2.0<br>1.8 | 4.5<br>3.2<br>2.1<br>0.5 | 2.6<br>1.1<br>0.8 | | |
| pMB1[b] | | | | | 2.75[c]<br>2.7[d] | |
| pMB1 | | | | | | 2.82[e]<br>1.16<br>0.7<br>0.5<br>0.25 |
| pCB61[f] | | 1.7 | | | | |
| *D. melanogaster* | | | | | | 2.3[g]<br>4 |

[a] *Hin*cII site located in the β lactamase ($Ap^r$) gene.
[b] pMB1 *is a clinical isolate plasmid that carries the EcoRI restriction and modification* genes as well as the colicin E1 production and colicin E1 immunity genes (Betlach et al., 1976).
[c] pMB1 *Hin*cII fragment carrying the *Eco*RI restriction and modification genes (Greene et al., 1976, manuscript in preparation).
[d] pBM1 *Hin*cII fragment carrying colicin E1 production and colicin E1 immunity genes
[e] There are six *Pst*I fragments in pMB1. The sixth has a molecular weight of $0.046 \cdot 10^6$ *daltons and was not identified in the agarose gel screening procedure.*
[f] pCB61 is a pBR322 derivative plasmid carrying a $1.7 \cdot 10^6$ dalton fragment of DNA cloned in the *Hin*dIII site. This fragment was isolated from the TnA transposon, and possibly carries a gene(s) involved in the translocation of the ampicillin translocon (Covarrubias et al., unpublished observations).
[g] These fragments were cloned by the homopolymer extension technique in the *Pst*I site of pBR322 and can be recovered after *Pst*I digestion of the recombinant plasmid DNA (*A. Dugaiczyk, personal communication*).

is a direct substrate for terminal transferase. Secondly, by extending the *Pst*I site in the plasmid with poly dG and the DNA to be cloned with poly dC, it is possible to regenerate two *Pst*I sites after annealing and repairing these two species of DNA in vivo or in vitro.

By fllowing this procedure, dC tailed *Drosophila melanogaster* DNA has been successfully cloned into the dG tailed *Pst*I site of pBR322. Transformants were selected for $Tc^r$ and screened for $Ap^s$ phenotypes. Two out of five *Drosophila melanogaster* DNA fragments cloned in this way had restored *Pst*I sites (Table II).

*(b)* 2. Cloning in the *Hin*cII sites of pBR322. The *Hin*cII restriction endonuclease which generates blunt-ended DNA fragments, recognizes two sites on pBR322. One of these sites is located in the $Tc^r$ and is also recognized by the *Sal*I endonuclease, while the other site is located in the $Ap^r$ gene, $0.17 \cdot 10^6$ daltons from the *Pst*I site. To demonstrate the cloning of blunt-ended DNA fragments into the *Hin*cII site of the $Ap^r$ gene, we used *Hin*cII digested pMB1 DNA which consisted of two fragments, $2.7 \cdot 10^6$ daltons and $2.75 \cdot 10^6$ daltons (P. Greene, unpublished observations). In order to preferentially cleave the *Hin*cII site in the $Ap^r$ gene, pBR322 DNA was first cleaved with *Sal*I endonuclease followed by a *Hin*cII endonuclease digestion. These digestions generated two DNA fragments each possessing one cohesive end (*Sal*I site) and one blunt end (*Hin*cII site). The pMB1 and pBR322 DNA were ligated under conditions to promote blunt-end ligation (see MATERIALS AND METHODS) and transformed into *E. coli* RRI. After selecting $Tc^r$ transformants and screening for $Ap^s$, two out of four $Tc^rAp^s$ transformants were found to contain recombinant plasmids carrying each of the two *Hin*cII fragments of pMB1. The two remaining $Tc^rAp^s$ transformants were found to carry pBR322 plasmids with no detectable change in molecular weight but only one *Hin*cII site equivalent to the *Sal*I site. We believe that these plasmids may result from in vivo recombination at the unligated *Hin*cII termini leading to the loss of both the *Hin*cII site and $Ap^r$.

*IV. Properties of pBR322 in the E. coli strain X1776*

The plasmids pBR322 and pBR313 could be used in *E. coli* X1776 as an EK2 system if it can be demonstrated that these vectors do not affect the survivability of *E. coli* 1776 (National Institutes of Health, USA, Recombinant DNA Research Guidelines, 1976). Therefore, pBR322 and pBR313 were transformed to the *E. coli* strain X1776 and examined for cell death in medium devoid of DAP, thymine and medium containing bile salts. DAP-less death of *E. coli* X1776 harboring these plasmids is as efficient as in that shown for *E. coli* X1776 alone (Fig. 5). Table III shows the plating efficiencies of the strains X1776 with and without the plasmids on media containing increasing concentrations of bile salts or lacking thymine. As in the DAP-less experiments, neither the pBR322 nor pBR313 affects the plating efficiency of X1776 under these conditions.

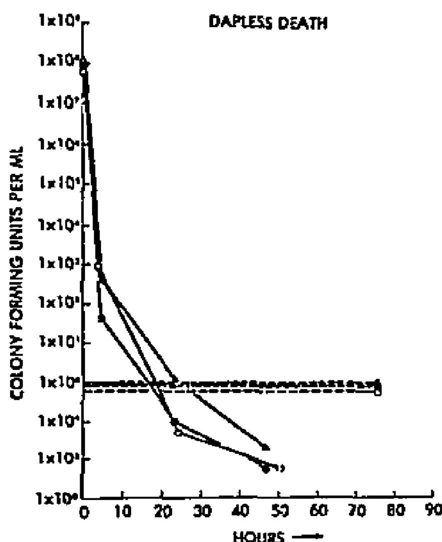


Fig. 5. DAP-less death of X1776, X1776(pBR313) and X1776(pBR322). For each of the strains the following procedure was performed. 500 ml early log cultures (~5 · $10^7$ to 1 · $10^8$ cells/ml) grown in LB + DAP (200 µg/ml) + Thy (50 µg/ml) were centrifuged. The cells were washed with BSG* and resuspended in 5 ml BSG. The resuspended cells were used to inoculate $DAP^-$ medium (LB + Thy − NaCl) and $DAP^+$ medium (LB + Thy + NaCl) using 0.5 ml of cells to 100 ml medium in each of 10 separate 250 ml erlenmeyer flasks. Cultures were incubated at 37° C with shaking. Samples were removed at various times from a fresh flask each time in order to reduce risk of contamination. The samples were titered on LB agar + DAP + Thy. Time points at 0.2 and 5 h were taken on the control cells grown in medium with DAP to verify their continued growth. At 24 h and later, cells were concentrated by filtering and the Millipore filters placed on LB agar. The dotted line indicates the titer at which the number of colony-forming unit have decreased by $10^8$ from the original titer. △—△, X1776 alone; ○—○, X1776 (pBR313); ●—●, X1776 (pBR322).

Data presented in Table IV show the mobilization of pBR313 and pBR 322 by de-repressed FI or FII R plasmids from "wild-type" *E. coli* K12 C600. In this case both plasmids are co-resident in the same cell and are mated directly with a wild-type *E. coli* K12 recipient cell line, SF185. As shown in Table IV, one can demonstrate that if F is coresident with wild-type ColE1, at the time of conjugation, ColE1 is mobilized at a frequency of about 0.5/recipient cell in 24 h. When F-Km or Rldrd19 are co-resident with pBR313 or pBR322, it is clear that mobilization occurs at a very low frequency. It is also clear that, relative to ColE1, pBR313 and pBR322 are less likely to be directly mobilized from either wild-type *E. coli* K12 or X1776 by de-repressed F-like plasmids.

Table V shows the transfer of pBR322 and pBR313 from *E. coli* C600 and *E. coli* X1776 in triparental matings using conjugative plasmids of various

TABLE III
PLATING EFFICIENCIES OF STRAIN X1776 (pBR313) AND X1776 (pBR322) ON MEDIA CONTAINING BILE SALTS OR WITHOUT THYMINE

Cultures were grown with shaking at 37°C in LB containing DAP (200 μg/ml) and thymine (50 μg/ml) to about 1—2 · $10^{9}$/ml. Cellswere pelleted by centrifugation, washed with BSG and concentrated 100-fold in BSG. Dilutions were made in growth medium and plated on the appropriate plates. Plates were incubated at 37°C.

| Medium | Plating efficiency[a] | | |
|---|---|---|---|
| | X1776 | X1776 (pBR313) | X1776 (pBR322) |
| L agar + DAP + Thy + 0.15% bile salts | $<6.25 \cdot 10^{-6}$ | $<7.14 \cdot 10^{-6}$ | $2.0 \cdot 10^{-7}$ |
| L agar + DAP + Thy + 0.37% bile salts | $<6.25 \cdot 10^{-8}$ | $4.29 \cdot 10^{-9}$ | $2.87 \cdot 10^{-8}$ |
| L agar + DAP + Thy + 0.75% bile salts | $<6.25 \cdot 10^{-9}$ | $2.6 \cdot 10^{-9}$ | $2.4 \cdot 10^{-8}$ |
| M9CAA agar + DAP + biotin (0.5 μg/ml) | $2 \cdot 10^{-9}$ | $<7.14 \cdot 10^{-8}$ | $6 \cdot 10^{-9}$ |

[a] Plating efficiency was calculated using the titer of X1776, X1776 (pBR313) or X1776 (pBR322) on L agar + DAP + Thy (without bile salts).

incompatibility groups. Since the FII, I and N compatibility groups comprise over 85% of the conjugative plasmids found in *E. coli* of healthy humans and animals, we focused upon these and two additional conjugative plasmids. Given the results shown in Table IV, it is not surprising that we could not detect ($<10^{8}$ per final recipient cell in 24 h) any instance for the mobilization of pBR313 and pBR322 from either C600 or X1776 in triparental matings. As a control an intermediate strain carrying the non-conjugative plasmid pRSF2124 was included. pRSF2124, a ColE1 derivative to which the $Ap^r$ gene has been transposed, could be mobilized by F-Km at a frequency of $10^{-4}$ per final recipient cell within 4 h.

## DISCUSSION

A new amplifiable cloning vehicle, the plasmid pBR322, which improves the cloning characteristics of the parental plasmid pBR313 (Bolivar et al., 1977), has been constructed by in vitro recombination techniques.

pBR322 was constructed in order to have a plasmid cloning vector with a single *Pst*I site in addition to those unique restriction sites already present in pBR313. Of the three *Pst*I sites in pBR313, the one located in the $Ap^r$ gene provided the most useful site for cloning purposes. The advantages of having a single *Pst*I site located in the $Ap^r$ gene are the following: (1) molecular cloning of *Pst*I endonuclease digested DNA fragments into the *Pst*I site will lead to the inactivation of the $Ap^r$ gene thus allowing for the detection of cells carrying recombinant plasmids by means of their $Ap^s$-$Tc^r$ phenotypes; (2) the benefits for molecular cloning by means of homodeoxypoly-

TABLE IV

MOBILIZATION OF pBR313 AND pBR322 by triparental mating[a]

| *E. coli* K12 donor | R plasmid | Inc. group | Drug resistance | Donor cells ml(·10[illegible]) | C600 1 h pBR313 | C600 1 h pBR322 | C600 24 h pBR313 | C600 24 h pBR322 | C600 control 24 h pRSF2124 | X1776 control 4 h pRSF2124 | X1776 1 h pBR313 | X1776 1 h pBR322 | X1776 24 h pBR313 | X1776 24 h pBR322 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| J5-3 | F-Km | FI | Km$^r$ | 3 | <10[illegible] | <10[illegible] | <10[illegible] | 10[illegible] | 2·10[illegible] | N.D.[b] | <10[illegible] | <10[illegible] | <10[illegible] | <10[illegible] |
| J5-3 | Rldrd | FII | Km$^r$, Su$^r$, Ap$^r$, Sm$^r$, Cm$^r$ | 1 | <10[illegible] | <10[illegible] | <10[illegible] | 10[illegible] | N.D. | <10[illegible] | <10[illegible] | <10[illegible] | <10[illegible] | <10[illegible] |
| J5-3 | R144 | I | Km$^r$Tc$^r$ | 5 | <10[illegible] | <10[illegible] | <10[illegible] | 10[illegible] | <10[illegible] | N.D. | <10[illegible] | <10[illegible] | <10[illegible] | <10[illegible] |
| J5-3 | N3 | N | Tc$^r$, Sm$^r$ Su$^r$ | 5 | <10[illegible] | <10[illegible] | <10[illegible] | 10[illegible] | <10[illegible] | <10[illegible] | <10[illegible] | <10[illegible] | <10[illegible] | <10[illegible] |
| J5-3 | Sa | W | Sm$^r$, Su$^r$, Cm$^r$, Km$^r$ | 2 | <10[illegible] | <10[illegible] | <10[illegible] | 10[illegible] | <10[illegible] | N.D. | <10[illegible] | <10[illegible] | <10[illegible] | <10[illegible] |
| J5-3 | RSF1040 | X | Ap$^r$ | 4.8 | <10[illegible] | <10[illegible] | <10[illegible] | 10[illegible] | <10[illegible] | N.D. | <10[illegible] | <10[illegible] | <10[illegible] | <10[illegible] |

[a] A triparental mating is one in which the conjugative plasmid resides in one strain (donor), the nonconjugative plasmid to be tested for mobilization in another (intermediate) and a third strain carrying a chromosomal *marker serves as the final recipient. Selection is made for the resistance markers present* on the nonconjugative plasmid and the chromosome of the final recipient. The intermediate strains were *E. coli* K12 either C600 or X1776 containing the cloning vehicle pBR313 or pBR322. The final recipient was *E. coli* K12 W1485-1 Nx$^r$. Cultures were grown at 37°C in LB plus DAP and thymine. Matings were done by addition to a 250 ml flask of 5 ml each of a culture of the donor strain containing the conjugative R plasmid either C600 or X1776 containing either pBR313 or pBR322 and the final recipient W1485-1. Matings were carried out without shaking for the indicated periods of time. The mating mixtures were plated (0.1 ml) on MacConkey agar containing 20 µg/ml of either tetracycline or amphicillin. C600 (pRSF2124) was included as a control. pRSF2124 is already impaired in its mobilization as compared to ColE1.

Concentrations (cells/ml) of intermediate and final recipient strains:

| | C600 (pBR313) | C600 (pBR322) | C600 (pRSF2124) | *X1776 (pBR313)* | *X1776 (pBR322)* | *W1485-1* |
|---|---|---|---|---|---|---|
| Exp. 1 | 9.2 · 10[illegible] | 4.7 · 10[illegible] | 3.7 · 10[illegible] | 2 · 10[illegible] | 2 · 10[illegible] | 1 · 10[illegible] |

[b] N.D., not done.

meric extension are two-fold. Not only is the protruding 3′OH of the cleaved *Pst*I site a direct substrate for *N*-terminal transferase, but the insertion of C-tailed DNA into a G-tailed plasmid generates two *Pst*I sites after polymerization and ligation in vitro or in vivo. This will allow for the recovery of the cloned DNA fragment after digesting the recombinant plasmid with the *Pst*I endonuclease. In addition, the main properties of pBR313 are conserved in pBR322 in that the cloning of *Hin*dIII, *Sal*I and *Bam*I endonuclease generated fragments in pBR322 continues to inactivate the $Tc^r$ gene. Screening for Tc sensitivity and therefore recombinant plasmids can be imposed on the transformed culture. Cloning of *Pst*I fragments in the appropriate site of pBR322 also inactivates one of the antibiotic-resistance genes. In all of these cases recombinant plasmids in transformed cells possess only one functional antibiotic-resistant gene.

Although the relative positions of the *Eco*RI and *Hin*dIII sites appear to be the same in both pSC101 and pBR322, cloning into the *Eco*RI site of pBR322 does not affect the level of $Tc^r$ as reported for pSC101 (Tait et al., 1977). Cloning of DNA fragments with one *Eco*RI terminus and the second terminus generated by one of the four endonucleases mentioned above into the appropriately digested plasmid will inactivate the $Tc^r$ or $Ap^r$ function.

Recent experiments of Heyneker et al. (1976) have demonstrated the usefulness of molecular cloning by blunt-end ligation. Therefore, the presence in pBR322 of only two substrate sites recognized by the *Hin*cII endonuclease, which generates blunt-ended DNA fragments, makes this plasmid a potential vector for cloning by blunt-end ligation. Cloning by blunt-end ligation has been achieved in the *Hin*cII site of the $Ap^r$ gene when this site was preferentially cleaved by the *Hin*cII endonuclease by prior digestion of the *Hin*cII site in the $Tc^r$ gene with *Sal*I endonuclease.

A low frequency of plasmid transmissibility has been established by the National Institutes of Health, USA, Recombinant DNA Guidelines as one of the most important safety features of a plasmid cloning vector. On the basis of the data presented in this paper, we feel that pBR313 and pBR322 within *E. coli* X1776 constitutes an improved EK2 host-vector system which can be used for the cloning of a variety of DNAs. Although the mechanism for mobilizing non-conjugative plasmids has received little attention, it is interesting to note that the frequency of mobilization of pBR313, pBR322 and pMB9 (R. Curtiss III, personal communication) has been significantly reduced with respect to wild-type ColE1. It has been recently reported (Gordon Dougan, personal communication) that a gene associated with high mobilization frequency has been mapped on the wild-type ColE1 plasmid. Therefore, ColE1 derivatives which involve deletions (pVH51) and enzymatic rearrangements (pMB9, pBR313 and pBR322) may result in the loss or alteration of mobilization. Although transposition of the $Ap^r$ and $Tc^r$ genes of pBR313 and pBR322 cannot be ruled out, experiments designed to test for this possibility have proven negative (data not shown).

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REFERENCES

Betlach, M.C., Hershfield, V., Chow, L., Brown, W., Goodman, H.M. and Boyer, H.W., A restriction endonuclease analysis of the bacterial plasmid controlling the *Eco*RI restriction and modification of DNA, Fed. Proc., 35 (1976) 2037–2043.

Bolivar, F., Rodriguez, R., Betlach, M. and Boyer, H.W., Construction and characterization of new vehicles, I. Ampicillin-resistant derivatives of the plasmid pMB9, Gene, 2 (1977) 75–93.

Cameron, J.R., Panasenko, S.M., Lehmon, I.R. and Davis, R.W., In vitro construction of bacteriophage λ carrying segments of *Escherichia coli* chromosome: Selection of hybrids containing the gene for DNA ligase, Proc. Natl. Acad. Sci. USA, 72 (1975) 3416–3420.

Clewell, D.B., Nature of ColE1 plasmid replication in the presence of chloramphenicol, *J. Bacteriol.*, *110(1972) 667–676.*

Cohen, S.N., Chang, A.C.Y., Boyer, H.W. and Helling, R., Construction of biologically functional plasmids in vitro. Proc. Natl. Acad. Sci. USA, 70 (1973) 3240–3244.

Covey, C., Richardson, D. and Carbon, J., A method for the deletion of restriction sites in bacterial plasmid deoxyribonucleic acid, Mol. Gen. Genet., 145 (1976) 155–158.

*Danna, K.J., Sack, G.H. and Nathans, D., Studies of simian virus 40 DNA, VII. A cleavage* map of the SV40 genome, J. Mol. Biol., 78 (1973) 363–376.

Datta, N. and Richmond, M.D., The purification and properties of a penicillinase whose synthesis is mediated by an R-factor in *Escherichia coli*, Biochem. J., 98 (1966) 204.

Dugaiczyk, A., Boyer, H.W. and Goodman, H.M., Ligation of *Eco*RI endonuclease-generated DNA fragments into linear and circular structures, J. Mol. Biol., 96 (1975) 171.

Greene, P.J., Betlach, M.C., Goodman, H.M. and Boyer, H.W., The *Eco*RI restriction endonuclease, in Wickner, R.B. (Ed.), Methods in Molecular Biology, Marcel Dekker, New York, 1974, pp. 87–111.

Greene, P.J., Heyneker, H., Betlach, M.C., Bolivar, F., Rodriguez, R., Covarrubias, A., Fodor, I. and Boyer, H.W., General method for restriction endonuclease purification, Manuscript in preparation.

Gromkova, R. and Goodgal, S.H., Action of *Haemophilus* endodeoxyribonuclease on biologically active deoxyribonucleic acid, J. Bacteriol., 109 (1972) 987.

Guerry, P., LeBlanc, D.J. and Falkow, S., General method for the isolation of plasmid deoxyribonucleic acid, J. Bacteriol., 116 (1973) 1064-1066.

Hamer, D. and Thomas, C., Molecular cloning of DNA fragments produced by restriction endonucleases *Sal*I and *Bam*I, Proc. Natl. Acad, Sci. USA, 73 (1976) 1537–1541.

*Heffron, F., 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., Plasmid ColE1 as a molecular vehicle for cloning and amplification of DNA, Proc. Natl. Acad. Sci. USA, 71 (1974) 3455—3461.

Hershfield, V. , Boyer, H.W., Chow, L. and Helinski, D., Characterization of a mini-ColE1 plasmid, J. Bacteriol., 126 (1976) 447—453.

Heyneker, H.L., Shine, J., Goodman, H.M., Boyer, H.W., Rosenberg, J., Dickerson, R.E., Narang, S.A., Itakura, K., Lin, S. and Riggs, A.D., Synthetic *lac* operator DNA is functional in vivo, Nature, 263 (1976) 748—752.

Landy, A., Ruedisueli, E., Robinson, L., Foeller, C. and Ross, W., Digestion of deoxyribonucleic acids from bacteriophage T7, λ and ϕ80h with site-specific nucleases from *Haemophilus influenzae* strain Rc and strain Rd, Biochemistry, 13(1974) 2134—2141.

Levy, S. and McMurry, L., Detection of an inducible membrane protein associated with R-factor-mediated tetracycline resistance, Biochem. Biophys. Res. Commun., 56 (1974) 1060—1068.

Lobban, P. and Kaiser, A.P., Enzymatic end-to-end joining of DNA molecules, J. Mol. Biol., 78 (1973) 453.

Morrow, J.F., Cohen, S.N., Chang, A.C.Y., Boyer, H.W., Goodman, H.M. and Helling, R.B., Replication and transcription of eukaryotic DNA in *Escherichia coli,* Proc. Natl. Acad. Sci. USA, 71 (1974) 1743—1747.

Panet, A., Van de Sande, J.H., Loewen, P.C., Khorana, H.G., Raae, A.J., Lillehaug, J.R. and Kleppe, K., Physical characterization and simultaneous purification of bacteriophage T4 induced polynucleotide kinase, polynucleotide ligase and deoxyribonucleic acid polymerase, Biochemistry, 12 (1973) 5045—5050.

Recombinant DNA Research Guidelines, National Institutes of Health, USA, Federal Register, 41, 1976, No. 131, 27901—27943.

Roberts, R.J., Myers, P.A., Morrison, A. and Murray, K., A specific endonuclease from *Arthrobacter luteus,* J. Mol Biol., 102 (1976) 157—165.

Rodriguez, R.L., Bolivar, F., Goodman, H.M., Boyer, H.W. and Betlach, M.C., Construction of new cloning vehicles, in Nierlich, D.P., Rutter, W.J. and Fox, C.F. (Eds.), Molecular Mechanisms in the Control of Gene Expression, Academic Press, New York, 1976, pp. 471—477.

Sgaramella, V., Van de Sande, J.H. and Khorana, H.G., A novel joining reaction catalyzed by T4 polynucleotide ligase, Proc. Natl. Acad. Sci. USA, 67 (1970) 1468—1475.

Smith, R., Blattner, R.F. and Davis, A., The isolation and partial characterization of a new restriction endonuclease from *Providencia stuartii,* Nucleic Acids Res. Commun., 3 (1976) 343.

So, M., Gill, R. and Falkow, S., The generation of a ColE1-Ap$^r$ cloning vehicle which allows detection of inserted DNA, Mol. Gen. Genet., 142 (1976) 239—249.

Sugino, A., Cozarelli, N.R., Heyneker, H.L., Shine, J., Boyer, H.W. and Goodman, H.M., Interaction of bacteriophage T4 RNA and DNA ligases in the joining of duplex DNA at base-paired ends, J. Biol. Chem. (1977) in press.

Tait, R.C., Rodriguez, R.L. and Boyer, H.W., Altered tetracycline resistance in pSC101 recombinant plasmids, Mol. Gen. Genet., 151 (1977) 327—331.

Wilson, G.A. and Young, F.F., Isolation of a sequence-specific endonuclease (*Bam*I) from *Bacillus amyloliquefaciens* H, J. Mol. Biol., 97 (1975) 123—126.

Yoshimori, R., Roulland-Dussoix, D., and Boyer, H.W., R-factor controlled restriction and modification of deoxyribonucleic restriction mutants, J. Bacteriol., 112 (1972) 1275—1283.

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