CONSTRUCTION AND CHARACTERIZATION OF NEW CLONING VEHICLES
II. A MULTIPURPOSE CLONING SYSTEM
(Recombinant DNA; molecular cloning plasmid vector; EK2 host-vector system)

FRANCISCO BOLIVAR*, RAYMOND L. RODRIGUEZ*, PATRICIA J. GREENE, MARY C. BETLACH, HERBERT L. HEYNEKER*, HERBERT W. BOYER
Department of Biochemistry and Biophysics, University of California, San Francisco, Calif. 94143 (U.S.A.) and
JORGE H. CROSA and STANLEY FALKOW
Department of Microbiology, University of Washington, Seattle, Wash. 98195 (U.S.A.)

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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 PstI site, located in the ampicillin-resistant gene (ApR), in addition to four unique restriction sites, EcoRI, HindIII, BamHI and SalI. 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.

*Present addresses: (F.B.) Departamento de Biologia Molecular, Instituto de Investigaciones Biomedicas, Universidad Nacional Autonoma de Mexico, Mexico 20, D.F., Apdo Postal 70228; (H.L.H.) Department of Molecular Genetics, University of Leiden, Wassenaarseweg 64, Leiden (The Netherlands); (R.L.R.) Department of Genetics, Briggs Hall, University of California, Davis, CA 95616 (U.S.A.).

Abbreviations: ApR, ampicillin-resistant; CmR, chloramphenicol-resistant; ColEImm, colicin immunity; DAP, diaminopimelic acid; DTT, dithiothreitol; KmR, kanamycin-resistant; LB, Luria broth; NxR, nalidixic-resistant; SDS, sodium dodecyl sulfate; SmR, streptomycin-resistant; SuR, sulfonamide-resistant; TcR, 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 HindIII, BamHI, EcoRI, HpaI, SalI and SmaI restriction endonucleases (Bolivar et al., 1977). In the case of the HindIII, BamHI and SalI endonuclease cloning sites, the insertion of DNA fragments inactivates the Tcr 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 PstI cleavage site located in the Apr gene as well as two HindIII sites located in the Ap\(^r\) and Tc\(^r\) genes. The PstI site can be used for molecular cloning of DNA fragments via homodeoxy polymeric extension (Lobban and Kaiser, 1973) and the HindIII 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 lacY 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 lacY Str^t was used to prepare unmethylated plasmid DNA for EcoRII digestions (Yoshimori et al., 1972). E. coli K12 strain X1776 F^-tauA53 dapD8 merA1 supE42 Δ40(gal-uracB)\(\lambda^-\) minB2 malA25 thyA57 metC65 Δ29(bioH-asd) cysB2 cycA1 HsdR2 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% KH₂PO₄, 0.06% Na₂HPO₄ 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 (Bet-lach et al., 1976).

(d) Enzymes

All the restriction enzymes used in this work, except for HpaI (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 MgCl₂, 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 CaCl₂ were added to 200 μl of CaCl₂-treated cells (5 • 10⁹ 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 at 37°C for 3 to 4 h until the
<table>
<thead>
<tr>
<th>Endonucleases</th>
<th>Substrate site</th>
<th>Reference</th>
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<th>Substrate site</th>
<th>Reference</th>
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<tbody>
<tr>
<td><em>AluI</em></td>
<td>AGCT</td>
<td>Roberts et al., 1976</td>
<td><em>HaeIII</em></td>
<td>GGC</td>
<td>Roberts et al., unpublished observations</td>
</tr>
<tr>
<td><em>BamHI</em></td>
<td>GATCC</td>
<td>Wilson and Young, 1975</td>
<td><em>HincII</em></td>
<td>GPTPyPuAC</td>
<td>Landy et al., 1974</td>
</tr>
<tr>
<td><em>BglII</em></td>
<td>...</td>
<td>Wilson and Young, unpublished observations</td>
<td><em>HindIII</em></td>
<td>A^1AGCTT</td>
<td>Danna et al., 1973</td>
</tr>
<tr>
<td><em>EcoRI</em></td>
<td>GAAATTC</td>
<td>Greene et al., 1976</td>
<td><em>HpaI</em></td>
<td>GTTAAAC</td>
<td>Gromkova and Goodgal, 1972</td>
</tr>
<tr>
<td><em>EcoRII</em></td>
<td>CCAAG G</td>
<td>Yoshimori et al., 1975</td>
<td><em>PstI</em></td>
<td>CTGCA^1G</td>
<td>Smith et al., 1976</td>
</tr>
<tr>
<td><em>HaeII</em></td>
<td>PuGCGC^1Py</td>
<td>Roberts et al., unpublished observations</td>
<td><em>SalI</em></td>
<td>GTCGAC</td>
<td>Bolivar et al., 1977</td>
</tr>
</tbody>
</table>
culture reached an absorbance of 0.5 to 0.6 A₆₀₀. 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 CaCl₂ (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 CaCl₂ 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 • 10⁶ dalton Ap⁵ Tc⁰ 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 PstI sites was located in the Ap⁵ gene. Therefore molecular cloning into this PstI site would result in recombinant molecules which could be detected by screening for Ap⁵ phenotypes. In order to construct a molecular cloning vector with one PstI site in the Ap⁵ gene, it was necessary to construct two derivatives of pBR313. An Ap⁵ Tc⁰ Col<IMM> plasmid, pBR318, containing one PstI site was obtained by transforming E. coli RRI with ligated PstI fragments of pBR313 and selecting for Tc⁰ transformants. Tc⁰ transformants which were Ap⁵ were found to carry plasmids that lack the 1.25 and 0.42 • 10⁶ dalton PstI fragments present in pBR313 (Fig.1). Another pBR313 derivative, pBR320, an Ap⁵ Tc⁰ Col<SM> plasmid with a molecular weight of 1.95 • 10⁶ daltons was obtained by transforming E. coli RRI with unligated EcoRII fragments of pBR313 and selecting for Ap⁵ transformants. Sixteen Ap⁵ Tc⁰ clones were examined, and one was found to carry a plasmid, pBR320, containing only one PstI 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 Ap⁵ Tc⁰ markers in a single low molecular weight relaxed plasmid containing one PstI substrate site. The construction of this plasmid was accomplished by the digestion of pBR318 with PstI and HpaI endonucleases which resulted in two pieces of DNA with molecular weights of 1.95 and 2.2 • 10⁶ daltons; the smaller DNA fragment carried the Tc⁰ gene(s) (Rodriguez et al., 1976; Tait et al., 1976; Bolivar et al., 1977) and part of the Ap⁵ gene as shown in Fig. 1. The plasmid pBR320 was cleaved with the restriction enzymes PstI and HincII to yield three fragments of DNA. The largest fragment, 1.15 • 10⁶ daltons, carries the "origin" of replication and the remaining portion of the Ap⁵ gene not present in the 1.95 • 10⁶ 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 PstI and EcoRII endonuclease digestions respectively. These two plasmids were separately digested with PstI and HpaI endonucleases (pBR318) and with PstI and HincII endonucleases (pBR320). The heavy lined regions from the PstI to HpaI sites in pBR318 and from PstI to HincII sites (Sall) 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 \textit{E. coli} RRI. Since neither pBR320 nor the 1.95 \( \cdot \) 10^6 dalton fragment of pBR318 carry the colicin E1 immunity gene, transformants were selected for Ap^r Te^r and then screened for sensitivity to colicin E1. This transformation yielded numerous Ap^r Te^r Col\(^S\) clones which carried plasmids (e.g. pBR321) with a molecular weight of 3.1 \( \cdot \) 10^6 daltons. As expected, this plasmid resulted from the addition of the 1.95 \( \cdot \) 10^6 dalton HpaI-PstI fragment of pBR318 and the 1.15 \( \cdot \) 10^6 d. PstI-HincII DNA piece from pBR320.

From this transformation we obtained in one instance a smaller, 2.6 \( \cdot \) 10^6 dalton, Ap^r Te^r Col\(^S\) plasmid. This plasmid, possibly the result of in vivo recombination event near unligated termini, was missing 0.5 \( \cdot \) 10^6 daltons of DNA from a region of pBR321, not associated with Ap^r, Te^r or DNA replication (Fig. 2). Because of its lower molecular weight compared with pBR321, pBR322 was chosen for further characterization of number and position of restriction sites.

\textbf{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 BamHI, EcoRI, HindIII, PstI and SalI 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 HincII sites in pBR322, one located 0.17 \( \cdot \) 10^6 daltons from the PstI site in the Ap^r gene and the other in Te^r gene, which is also a SalI site (Bolivar et al., 1977).

The EcoRII restriction endonuclease was used to further characterize pBR322. As shown in Fig. 4a (slot 3), pBR322 has five EcoRII sites which yield fragments of 1.25, 0.64, 0.53, 0.22 and 0.04 \( \cdot \) 10^6 daltons upon digestion. Slots 3, 4, 5, 6 and 7 (Fig. 4a) show respectively, EcoRI, HincII, SalI, BamHI and BglI digestions of EcoRII-digested pBR322 DNA. In the case of the EcoRI endonuclease digest, the largest EcoRII fragment when cleaved gives two new fragments of 0.08 and 1.07 \( \cdot \) 10^6 daltons. Slots 5 and 6 show that SalI and BamHI endonucleases cleave the same 0.53 \( \cdot \) 10^6 dalton EcoRII fragment as in pBR313 (Bolivar et al., 1977) and generates 0.29 and 0.24 \( \cdot \) 10^6 dalton DNA fragments after the SalI endonuclease digestion and 0.4 and 0.13 \( \cdot \) 10^6 dalton fragments after BamHI endonuclease digestion. Slot 4 shows that the same 0.53 \( \cdot \) 10^6 dalton EcoRII piece is cleaved by HincII endonuclease, generating the same 0.29 and 0.24 \( \cdot \) 10^6 dalton fragments that the SalI endonuclease produces. The 1.25 \( \cdot \) 10^6 dalton EcoRII fragment is also cleaved by HincII endonuclease into 0.82 and 0.43 \( \cdot \) 10^6 dalton fragments. Slot 7 shows the double digestion pattern of pBR322 DNA using EcoRII and BglI endonucleases. Three EcoRII fragments are cleaved by BglI endonuclease into smaller pieces. The largest EcoRII fragment (1.25 \( \cdot \) 10^6 daltons) is cleaved into 0.57 and 0.67 \( \cdot \) 10^6 dalton fragments. BglI endonuclease also cleaves the 0.53 \( \cdot \) 10^6 dalton fragments into 0.47 and 0.06 \( \cdot \) 10^6 dalton
Fig. 2. *Hinf*I-*Eco*RI endonuclease analyses of pBR321 and pBR322. Molecular weight estimates are based in the seven PM2 fragments generated by the *Hind*III endonuclease (31.4, 13.4, 6.0, 3.1, 2.9, 1.4, and 0.6, the last one not seen in the gel, Wes Brown, personal communication) (slot 1). The *Hinc*I endonuclease and *Hinc*I-*Eco*RI endonuclease single and double digestions of pBR322 are shown in slots 3 and 2 respectively while the *Hinc*I endonuclease and *Hinc*I-*Eco*RI endonuclease digestions of pBR321 are shown in slots 5 and 4 respectively. It can be seen that the $0.64 \times 10^4$ *Hinc*I 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$^c$ or Tc$^c$ genes nor the region located in the small *Hinc*I fragment ($0.64 \times 10^4$ 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 $1 \times 10^4$ daltons (outer circle) and 0.1 kilobases (inner circle). The estimated size of the Ap and Tc 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, 1965) and the Tc-associated proteins detected in the minicell system (Levy and McMurry, 1974; Tait et al., 1976).

Positioning the left-hand boundary of the Tc gene was based on our knowledge that cloning into the EcoRI site of pBR313 did not affect Tc while cloning into the HindIII site did affect the Tc mechanism. The position and size of the Tc region is also consistent with the orientation of the TnA in pBR25 (Bolivar et al., 1977). Only ten out of twelve HaeII and AluI and seven out of seventeen HaeIII substrate sites are represented on the circular map of pBR322. The position of two of the ten AluI sites located at $1.8 \times 10^4$ daltons and $1.86 \times 10^4$ daltons were mapped on a $0.7 \times 10^4$ dalton plasmid which encompasses this region of pBR322 (unpublished observation).
Fig. 4a. Acrylamide gel electrophoresis of EcoRII-cleaved pBR322 DNA. Analysis of EcoRII endonuclease (slot 2) and double endonuclease EcoRII-EcoRI digestions (slot 3), EcoRII-HincII (slot 4), EcoRII-SalI (slot 5), EcoRII-BamHI (slot 6) and EcoRII-BgII (slot 7) of pBR322 DNA. The seven HindIII-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 - 10^6 dalton EcoRII fragment is cleaved by BgII endonuclease into 0.16 and 0.06 - 10^6 dalton DNA fragments. These data allow us to localize four EcoRII sites in the pBR322 map (Fig. 3).

The AluI endonuclease cleaves pBR322 into approximately 12 fragments (Fig. 4b, slot 2). Four AluI sites were mapped by analysis of the molecular weights of DNA fragments generated by double endonuclease digestion (Fig. 3). By comparing the AluI fragments of pBR318 and pBR320 (data not shown), we have tentatively localized four additional AluI sites in the map of pBR322. The AluI site located between the EcoRI and the HindIII 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 HaeII sites and the 3 BgII 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 HindIII, BamHI and SalI sites are present
Fig. 4b. Acrylamide gel electrophoresis of AluI endonuclease digested pBR322 DNA. Analysis of AluI endonuclease (slot 2) and double digestions of AluI-EcoRI (slot 3), AluI-BamHI (slot 4), AluI-SalI (slot 5), AluI-HindII (slot 6), and AluI-PstI (slot 7) endonucleases of pBR322 DNA. The seven HindIII 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 AluI endonuclease generated fragment (0.43 \( \times \) 10^6 daltons) is cleaved by the EcoRI endonuclease generating a 0.42 \( \times \) 10^6 daltons band and a 0.01 \( \times \) 10^6 dalton fragment not seen in the gel. BamHI endonuclease cleaves the 0.38 \( \times \) 10^6 dalton fragment into two fragments of 0.2 and 0.18 \( \times \) 10^6 daltons. SalI endonuclease cleaves the same 0.33 \( \times \) 10^6 dalton AluI fragment generating a piece of 0.34 \( \times \) 10^6 daltons and a small one of 0.04 \( \times \) 10^6 daltons (not seen in the gel). HindII endonuclease cleaves two AluI fragments; cleaves in the SalI site generating the same 0.34 \( \times \) 10^6 dalton AluI 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 HindIII, BamHI and SalI restriction endonucleases and cloned into their respective sites in pBR322. These recombinants were Ap\( ^r \)Tc\( ^r \) (Table II). Insertion of DNA fragments into the EcoRI 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 PstI site in pBR322 is located in the Ap\( ^r \) gene, we have cloned several fragments of DNA in PstI site of this plasmid (see Table II). PstI generated fragments of the plasmid pMB1 (Betlach et al., 1976) were
ligated into the PstI site of pBR322 and transformed into RRI. Transformants were selected for Tc r and screened for Ap s phenotypes. This experiment resulted in the cloning of five different PstI fragments representing nearly the whole pMB1 genome (Table II).

The unique PstI site in pBR322 provides two advantages for the molecular cloning of DNA by means of the homodeoxypolymeric DNA extension technique (Lobban and Kaiser, 1973). First, the PstI site, which has the sequence C T G C A T G (Smith et al., 1976) provides a protruding 3'OH which

**TABLE II**

**MOLECULAR CLONING OF VARIOUS DNA FRAGMENTS IN pBR322**

<table>
<thead>
<tr>
<th>Sources</th>
<th>Restriction endonuclease substrate site</th>
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<tr>
<td></td>
<td>EcoRI</td>
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<tr>
<td>N. crassa</td>
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<td></td>
<td>1.1</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>pCB61f</td>
<td></td>
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<tr>
<td>D. melanogaster</td>
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</table>

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 HindII fragment carrying the EcoRI restriction and modification genes (Greene et al., 1976, manuscript in preparation).
d. pBM1 HindII fragment carrying colicin E1 production and colicin E1 immunity genes.
e. There are six PstI fragments in pMB1. The sixth has a molecular weight of 0.046 - 10^6 daltons and was not identified in the agarose gel screening procedure.
f. pCB61 is a pBR322 derivative plasmid carrying a 1.7 • 10^6 dalton fragment of DNA cloned in the HindIII 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 PstI site of pBR322 and can be recovered after PstI digestion of the recombinant plasmid DNA (A. Dugaiczky, personal communication).
is a direct substrate for terminal transferase. Secondly, by extending the PstI site in the plasmid with poly dG and the DNA to be cloned with poly dC, it is possible to regenerate two PstI sites after annealing and repairing these two species of DNA in vivo or in vitro.

By following this procedure, dC tailed *Drosophila melanogaster* DNA has been successfully cloned into the dG tailed PstI 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 PstI sites (Table II).

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

\[\text{IV. Properties of pBR322 in the } E. coli \text{ strain X1776}\]

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 10^6 from the original titer. 4—4, X1776 alone; o—o, X1776 (pBR313); 3—3, X1776 (pBR322).

Data presented in Table IV show the mobilization of pBR313 and pBR322 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 × 10⁸/ml. Cells were 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.

<table>
<thead>
<tr>
<th>Medium</th>
<th>X1776</th>
<th>X1776 (pBR313)</th>
<th>X1776 (pBR322)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L agar + DAP + Thy + 0.15% bile salts</td>
<td>&lt;6.25 × 10⁻⁶</td>
<td>&lt;7.14 × 10⁻⁶</td>
<td>2.0 × 10⁻⁷</td>
</tr>
<tr>
<td>L agar + DAP + Thy + 0.37% bile salts</td>
<td>&lt;6.25 × 10⁻⁴</td>
<td>4.29 × 10⁻⁷</td>
<td>2.87 × 10⁻⁸</td>
</tr>
<tr>
<td>L agar + DAP + Thy + 0.75% bile salts</td>
<td>&lt;6.25 × 10⁻⁸</td>
<td>2.6 × 10⁻⁷</td>
<td>2.4 × 10⁻⁹</td>
</tr>
<tr>
<td>M9CAA agar + DAP + biotin (0.5 µg/ml)</td>
<td>2 × 10⁻⁹</td>
<td>&lt;7.14 × 10⁻⁸</td>
<td>6 × 10⁻⁵</td>
</tr>
</tbody>
</table>

* 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 (<10⁸ per final recipient cell in 24 h) any instance for the mobilization of pBR313 and pBR322 from either C600 or X1776 in tripaperal matings. As a control an intermediate strain carrying the non-conjugative plasmid pRSF2124 was included. pRSF2124, a CoIE1 derivative to which the Ap⁺ 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 *PstI* site in addition to those unique restriction sites already present in pBR313. Of the three *PstI* sites in pBR313, the one located in the *Ap⁺* gene provided the most useful site for cloning purposes. The advantages of having a single *PstI* site located in the *Ap⁺* gene are the following: (1) molecular cloning of *PstI* endonuclease digested DNA fragments into the *PstI* site will lead to the inactivation of the *Ap⁺* gene thus allowing for the detection of cells carrying recombinant plasmids by means of their *Ap⁺-Tc⁺* phenotypes; (2) the benefits for molecular cloning by means of homodeoxypoly-
<table>
<thead>
<tr>
<th>E. coli</th>
<th>R plasmid</th>
<th>Inc. group</th>
<th>Drug resistance</th>
<th>Donor cells ml(-10⁴)</th>
<th>C600 1 h</th>
<th>C600 24 h</th>
<th>C600 control 24 h</th>
<th>X1776 control 4 h</th>
<th>X1776 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>J5-3</td>
<td>F',Km</td>
<td>FI</td>
<td>Km⁷</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>10⁻⁸</td>
<td>N.D.</td>
<td>&lt;10⁻⁸</td>
</tr>
<tr>
<td>J5-3</td>
<td>Rbnd</td>
<td>FII</td>
<td>Km⁷,Su⁵, Ap⁵, Sm⁷, Km²</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>10⁻⁸</td>
<td>N.D.</td>
<td>&lt;10⁻⁸</td>
</tr>
<tr>
<td>J5-3</td>
<td>R144</td>
<td>L</td>
<td>Km⁷,Te⁴</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>10⁻⁸</td>
<td>N.D.</td>
<td>&lt;10⁻⁸</td>
</tr>
<tr>
<td>J5-3</td>
<td>N3</td>
<td>N</td>
<td>Te⁴,Sm⁷</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>10⁻⁸</td>
<td>N.D.</td>
<td>&lt;10⁻⁸</td>
</tr>
<tr>
<td>J5-3</td>
<td>Sa</td>
<td>W</td>
<td>Sm⁷,Sm⁷, Km¹</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>10⁻⁸</td>
<td>N.D.</td>
<td>&lt;10⁻⁸</td>
</tr>
<tr>
<td>J5-3</td>
<td>RSP1040 X</td>
<td>Ap⁵</td>
<td>4.8</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>&lt;10⁻⁸</td>
<td>10⁻⁸</td>
<td>N.D.</td>
<td>&lt;10⁻⁸</td>
</tr>
</tbody>
</table>

* 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 Na⁺. 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 X 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 ampicillin. C600 (pRSF3124) was included as a control. pRSF2124 is already impaired in its mobilization as compared to ColE1.

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

<table>
<thead>
<tr>
<th>C600 (pBR313)</th>
<th>C600 (pBR322)</th>
<th>C600 (pRSF2124)</th>
<th>X1776 (pBR313)</th>
<th>X1776 (pBR322)</th>
<th>W1485-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1</td>
<td>9.2 • 10⁸</td>
<td>4.7 • 10⁸</td>
<td>3.7 • 10⁸</td>
<td>2 • 10⁸</td>
<td>2 • 10⁸</td>
</tr>
</tbody>
</table>

b N.D., not done.
meric extension are two-fold. Not only is the protruding 3'OH of the cleaved PstI site a direct substrate for N-terminal transferase, but the insertion of G-tailed DNA into a G-tailed plasmid generates two PstI 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 PstI endonuclease. In addition, the main properties of pBR313 are conserved in pBR322 in that the cloning of HindIII, SalI and BamI 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 PstI 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 EcoRI and HindIII sites appear to be the same in both pSCI01 and pBR322, cloning into the EcoRI site of pBR322 does not affect the level of Tc\(^r\) as reported for pSCI01 (Tait et al., 1977). Cloning of DNA fragments with one EcoRI 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 HindII 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 HindII site of the Ap\(^r\) gene when this site was preferentially cleaved by the HindII endonuclease by prior digestion of the HindII site in the Tc\(^r\) gene with SalI 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


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.


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 Re and strain Rd, Biochemistry, 13(1974) 2134--2141.


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


Wilson, G.A. and Young, F.F., Isolation of a sequence-specific endonuclease (Bam1) 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 acid restriction mutants, J. Bacteriol., 112 (1972) 1275--1283.

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