Gene, 4 (1978) 121—136

CONSTRUCTION AND CHARACTERIZATION OF NEW CLONING VEHICLES
III. DERIVATIVES OF PLASMID pBR322 CARRYING UNIQUE *Eco* RI SITES FOR SELECTION OF *Eco* RI GENERATED RECOMBINANT DNA MOLECULES

(Cloning vehicle; restriction endonucleases; *Bam*HI; *Eco*RI; *Hinc*II; *Hin*dIII; *Pst*I; *Sal*I; *Xma*I; *Sma*I; *Bgl*II; *Dpn*II; colicin E1; tetracycline, ampicillin, chloramphenicol resistance)

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(Received February 14th, 1978)
(Accepted July 14th, 1978)

SUMMARY

In vitro recombinant DNA techniques were used to construct two new cloning vehicles, pBR324 and pBR325. These vectors, derived from plasmid pBR322, are relaxed replicating elements. Plasmid pBR324 carries the genes from pBR322 coding for resistance to the antibiotics ampicillin (Ap^r) and tetracycline (Tc^r) and the colicin E1 structural and immunity genes derived from plasmid pMBI. Plasmid pBR325 carries the Ap^r and Tc^r genes from pBR322 and the cloramphenicol resistance gene (Cm^r) from phage P1Cm. In these plasmids the unique *Eco*RI restriction site present in the DNA molecule is located either in the colicin E1 structural gene (pBR324) or in the Cm^r gene(pBR325). These vectors were constructed in order to have a single *Eco*RI site located in the middle of a structural gene which when inactivated would allow, for the easy selection of plasmid recombinant DNA molecules. These plasmids permit the molecular cloning and easy selection of *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I, *Hinc*II, *Sal*I, (*Xam*I), *Sma*I, (*Xma*I), *Bgl*II and *Dpn*II restriction generated DNA molecules.

INTRODUCTION

The most critical component of cloning techniques is the cloning vehicle. It is now clear that improved plasmid cloning vectors have made possible the molecular cloning of various interesting DNA molecules (Seeburg et al., 1977;

Abbreviation: DTT, dithiothreitol.

Shine et al., 1977; Ullrich et al., 1977; McCorkel et al., 1978) and in some cases the expression of such cloned information (Itakura et al., 1977; Vapnek et al., 1977). Three of the bacterial plasmids commonly used as cloning vectors are pMB9 (Rodríguez et al., 1977) and its ampicillin-resistant (Ap^r) derivatives pBR313 (Bolivar et al., 1977a) and pBR322 (Bolivar et al., 1977b). Plasmid pBR322 contains the Ap^r and the Tc^r genes derived from pRSF2124 (So et al., 1977) and pSC101 (Cohen et al., 1972) respectively in combination with replication elements of pMBI, a ColE1-like plasmid (Betlach et al., 1976; Bolivar et al., 1977c). The pBR322 plasmid provides single cleavage sites for the *Hin*dIII, *Bam*HI, *Sal*I, *Pst*I and *Eco*RI restriction endonucleases. In the case of the first three enzymes the insertion of DNA fragments inactivates the Tc^r gene(s). Molecular cloning into the unique *Pst*I site inactivates the Ap^r gene. Although the relative positions of the *Eco*RI and *Hin*dIII sites are the same in both pBR322 and pSC101 (Rodriguez et al., 1978), the insertion of material into the *Eco*RI site of pBR322 does not affect the level of Tc resistance which was reported to be altered in the case of pSC101 (Tait et al., 1977). Therefore cloning into the *Eco*RI site of pBR322 does not permit an easy selection of recombinant molecules as it does in the cases of the other aforementioned endonucleases.

This paper reports the construction and initial characterization of two different pBR322 derived plasmids containing unique *Eco*RI sites that allow for selection of *Eco*RI recombinant molecules. One of these plasmids, pBR324, carries the colicin E1 structural and immunity genes derived from pMBI. The position of a unique *Eco*RI site (and a unique *Sma*I site) in the colicin E1 structural gene provides an easy selection for *Eco*RI and *Xma*I (or *Sma*I) endonuclease-generated DNA fragments.

The second pBR322 derived plasmid, pBR325, carries the chloramphenicol resistant gene derived from phage $P1Cm^r$. This plasmid has a unique *Eco*RI site in the Cm^r gene; thus recovery of cells harboring *Eco*RI-derived recombinant DNA molecules is facilitated by virtue of their Ap^r, Tc^r, Cm^s phenotypes.

MATERIAL AND METHODS

Bacterial and phage strains

E. coli K-12 strain RR1 (*pro leu thi lac* Y *rpSL*20 *hsdR hsdM ara*-14, *gal* K2, *xyl*-5, *mtl*-1, *sup*E44, λ^-, F^-) was used as the recipient cell in the transformation experiments. *E. coli* strain GM48 (*dam*-3 *dcm*-6 *thr*-1 *leu*-6 *thi*-1 *lac*-Y *gal* K2 *gal* T22 *ara*-14 *tonA*-31 *tsx*-78 *sup*E44) was obtained from T. Kadish. *E. coli* strain JC411 (ColE1) and $PICm^r$ phage DNA were generously provided by F. Bastarrachea.

N. crassa DNA was a gift from R. Palacios.

Media and buffers

E. coli strain RRI was grown in either Luria Broth (LB) or M9 glucose

minimal media. When needed, ampicillin, tetracycline and chloramphenicol were added to LB plates at final concentrations of 25 μg/ml.

Preparation of plasmid DNA

Plasmid DNA (pBR322, pBR323 and pBR324) was prepared by amplifying logarithmically growing cultures by the addition of 170 μg/ml of chloramphenicol (Clewell et al., 1972). Spectinomycin (300 μg/ml) was used for the amplification of cells carrying the Cm^r plasmid pBR325.

Extraction and purification of plasmid DNA was achieved by the cleared lysate technique previously described by Betlach et al. (1976).

Enzymes

All the restriction enzymes used in this work, except *Xma*I (generously provided by P. Valenzuela), *Dpn*II (a gift from T. Kadish) and *Hha*I (Bethesda Research Laboratories), were purified according to the procedure of Greene et al. (1978). Reaction conditions for the various restriction endonucleases have been previously described (Bolivar et al., 1977a). T4 DNA ligase, a gift from H. Heyneker, was purified from T4amN32 infected *E. coli* B cells according to the procedure described by Panet et al. (1973).

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 were varied to promote polymerization or circularization. When blunt-ended DNA fragments were ligated, the concentration of ends was at least 0.2 μM and approx. 5 U. of T4 DNA ligase per ml were added to the reaction mixture (Heyneker et al., 1976). When DNA fragments with cohesive ends were ligated, 1 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).

Agarose and acrylamide gel electrophoresis

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

Transformation of E. coli K-12

E. coli RRI cells were prepared for transformation by the method described by Cohen et al. (1972).

Colicin E1 immunity and production tests

Colicin E1 was prepared from *E. coli* K-12 strain JC411 (ColE1). The cells were grown to log phase ($4 \cdot 10^8$ cells/ml) and treated with mitomycin C (2 μg/ml). 20 min later the culture was pelleted, resuspended in phosphate buffer (10 mM pH 7.4) and sonicated. The supernatant of a high-speed centrifugation (30 K/35 rotor Spinco/1 h/4°C) was resuspended in 10 mM

potassium phosphate pH 7.2, 1 mM NaN_3, 1 mM EDTA and stored in the same buffer containing 50% glycerol at −20°C. For colicin E1 immunity tests the culture to be tested was spread on LB plates and a drop of a 1/100 dilution of the above colicin E1 solution was added after allowing absorption of the cells. The cells growing in the center of the drop were retested for colicin E1 immunity. Colicin E1 production was determined by the method described by Herschman and Helinski (1967).

RESULTS

Plasmid pBR322 has been extensively mapped using a series of restriction endonucleases. Recently the nucleotide sequence of the Ap^r gene and of most of the Tc^r gene of the plasmid have been determined (G. Sutcliffe and W. Gilbert, personal communication); a nucleotide sequence of 1230 bp of a region comprising the origin of replication has also been determined (Bolivar et al., 1977c; 1978).

The unique *Eco*RI site in pBR322 is located 21 bp from the *Hin*dIII site, which has been reported to be localized in the Tc^r promoter (Rodríguez et al., 1977), and 207 bp from the putative AUG (fmet) of the Ap^r gene (G. Sutcliffe and W. Gilbert, personal communication). Therefore cloning into the *Eco*RI site of pBR322 (see Fig.1) does not affect the antibiotic resistant phenotype of cells carrying *Eco*RI recombinant molecules. The revised molecular weight of pBR322 has been determined to be 2.7 Mdaltons (unpublished observations).

It has been reported that the plasmids pMBI (Betlach et al., 1976) and ColE1 (Hershfield et al., 1974) carry unique *Eco*RI sites in the colicin E1 structural gene. Another small structural gene carrying a unique *Eco*RI site is the Cm^r gene present in phage P1Cm (unpublished observations). Two different cloning experiments were designed to obtain derivatives of pBR322 with unique *Eco*RI sites carrying either the colicin E1 structural gene or the Cm^r gene.

Construction of pBR323 and pBR324

Plasmid pMBI, although independently isolated, has extensive structural and functional homologies with the ColE1 plasmid. They share the genes for colicin E1 production and immunity as well as a series of well-defined replicative properties (Betlach et al., 1976; Bolivar et al., 1977c). They differ mainly due to the presence of an extra segment of DNA in pMBI which codes for the *Eco*RI enzymes (Yoshimore et al., 1972; Betlach et al., 1976). A simplified restriction endonuclease (*Sal*I) linear map is shown in Fig.1. It can be seen that the colicin E1 structural and immunity genes are located in a $2.8 \cdot 10^6$ dalton *Hin*cII fragment.

In order to construct a molecular cloning vector derived from pBR322 with a unique *Eco*RI site in the colicin E1 structural gene, plasmid pBR322 was digested with the restriction endonuclease *Eco*RI and the resulting DNA

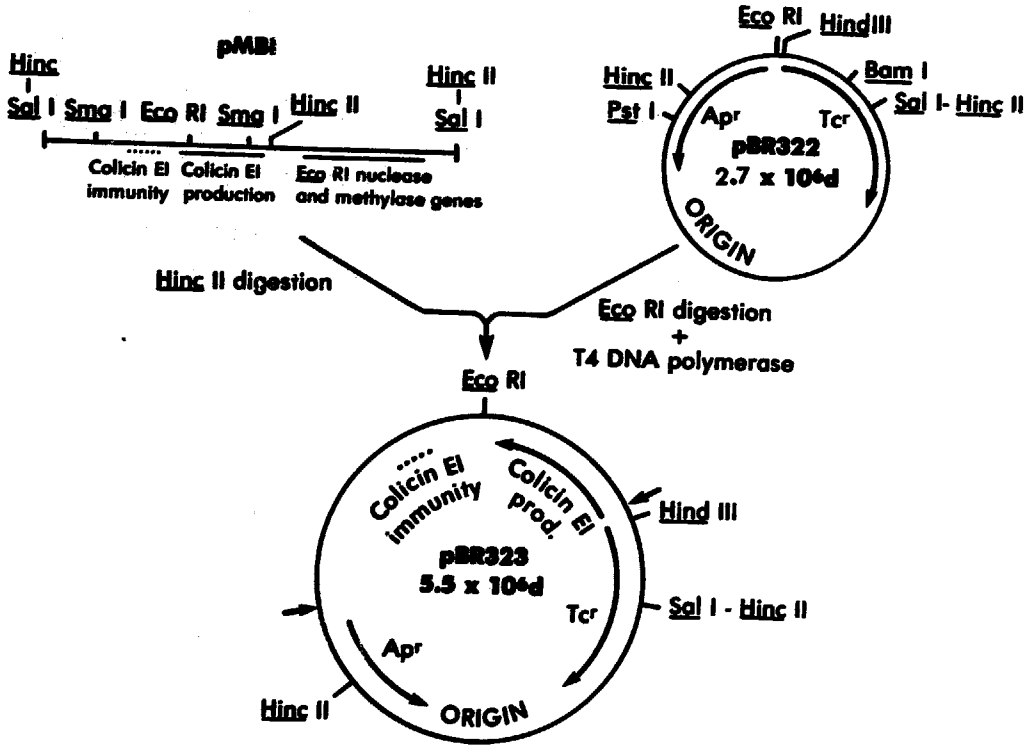

Fig.1. Diagrammatic representation of the construction of pBR323. The parental plasmid pBR322 was used for cloning a *Hinc*II restriction fragment that carries the colicin E1 structural and immunity genes derived from plasmid pMBI.

Plasmid pBR322 was digested with the restriction enzyme *Eco*RI and the linear DNA was treated with T4 DNA polymerase to generate a blunt-ended DNA fragment. This DNA was ligated to a *Hinc*II fragment derived from pMBI that carries the colicin E1 structural and immunity genes. The ligated mixture was used to transform *E. coli* strain RR1.

was treated with t4 DNA polymerase to fill the cohesive *Eco*RI generated ends. This DNA was then ligated with *Hinc*II digested pMBI DNA. When ligated, these termini do not result in the recovery of *Eco*RI or *Hinc*II sites. The Ap^r, Tc^r, colicin E1 immune and colicin E1 producing transformants, which occurred at a frequency of $5 \cdot 10^{-7}$/ml/μg DNA, were screened for plasmid DNA which gave a linear $5.5 \cdot 10^6$ dalton plasmid molecule upon digestion with *Eco*RI. Fig.2 shows the restriction patterns of one of the resulting plasmids, pBR323, which occurred at a frequency of approx. $8 \cdot 10^{-8}$ /ml/μg DNA. The data from Fig.2 are summarized in Fig.3.

As we expected, on agarose electrophoresis of its DNA digests, pBR323 was found to carry unique substrate sites for the *Hin*dIII, *Bam*HI, *Sal*I and *Eco*RI restriction enzymes. Double and triple digests of the plasmid (data not shown) showed that the relative positions of the first three sites were identical to those mapped in pBR322. As can be seen in Figs. 2 and 3, there are only two *Hinc*II sites in pBR323, one located in the Ap^r gene ($0.17 \cdot 10^6$

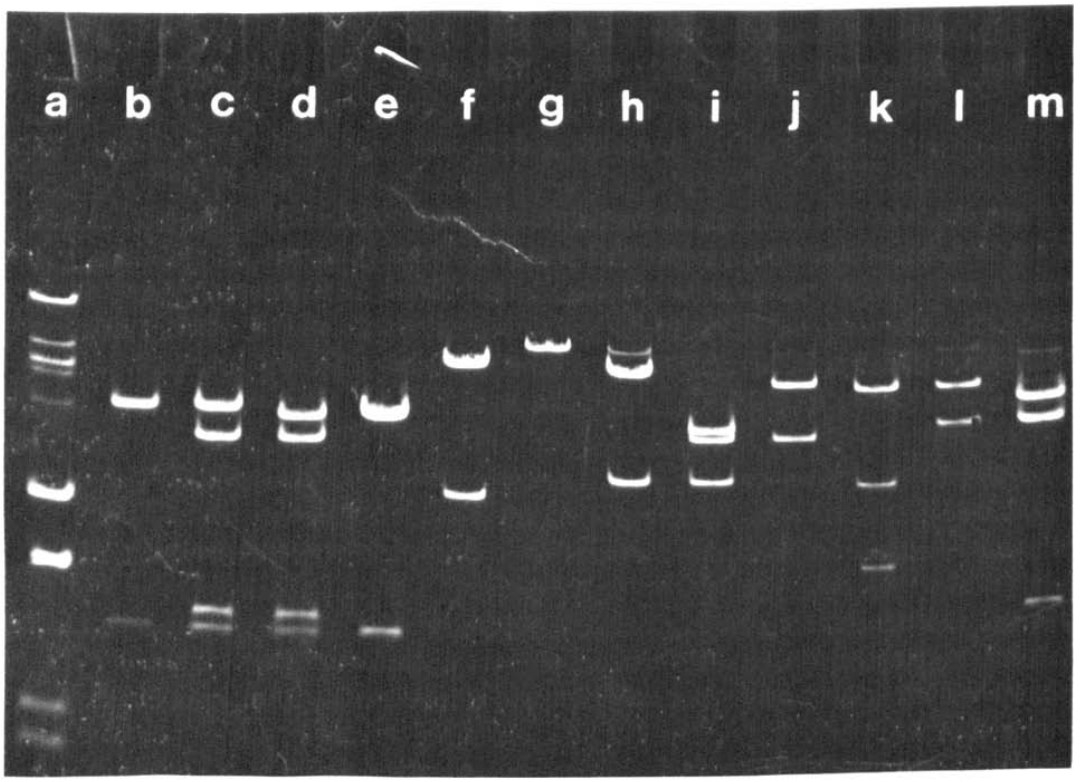

Fig.2. Agarose slab gel electrophoresis (1%) of plasmid pBR323 cleaved by *Eco*RI, *Bam*HI, *Sal*I, *Hin*cII, *Pst*I, and *Sma*I. Digested DNA (0.3—0.5 μg) was applied to the sample slots in 30 μl volumes. Agarose electrophoresis was carried out as previously described (Bolivar et al., 1977a). Molecular weight estimates (slot a) are based on the six λ fragments generated by *Eco*RI and the six *Hin*dIII-generated fragments of the SV40 genome. Slot g shows the *Eco*RI linear fragments of pBR323. Slot h shows the double (partial) digestion by *Eco*RI-*Sal*I; slot f shows the double (partial) digestion by *Eco*RI *Bam*HI. Slot j shows the *Hin*cII digestion of the plasmid DNA and slot i shows the double digestion by *Hin*cII-*Eco*RI of pBR323. The *Sma*I digestion (partial) is shown in slot l, while partial double digestion by *Sma*I-*Eco*RI and *Sma*I-*Sal*I are shown in slots m and k respectively. The restriction enzyme *Pst*I cleaves the plasmid pBR323 in five sites; however only three of these fragments are shown in this 1% gel. The two largest fragments are superimposed (slot b). As can be seen, *Sal*I cleaves one of the two largest *Pst*I fragments (slot c). The restriction enzyme *Hin*cII cleaves the two largest *Pst*I fragments (slot d). The double digestion *Pst*I-*Eco*RI is shown in slot e.

daltons from the *Pst*I site in the Ap^r gene) and the other, which is also the *Sal*I site in the Tc^r gene (Bolivar et al., 1977a,b).

The unique *Eco*RI site is located at $1.43 \cdot 10^6$ daltons (counterclockwise) from the *Sal*I site, $1.28 \cdot 10^6$ daltons from the *Bam*HI site and $1.1 \cdot 10^6$ daltons from the *Hin*dIII site (Fig.2, slots h and f, Fig.3). The *Sma*I restriction endonuclease was used to further characterize pBR323. As shown in Fig.2 slot l, pBR323 has two *Sma*I sites which yield fragments of 3.33 and $2.17 \cdot 10^6$ daltons. Slots *k* and *m* (Fig.2) show respectively *Eco*RI and *Sal*I digestions of *Sma*I digested pBR323 DNA. In the case of the *Eco*RI digest, the smallest *Sma*I fragment when cleaved generated two new fragments of

approx. 1.3 and $0.87 \cdot 10^6$ daltons. Slot *m* shows that the restriction enzyme *Sal*I cleaves the largest *Sma*I into two fragments of molecular weights of approx. 2.77 and $0.58 \cdot 10^6$ daltons. These results allow for the localization of the relative positions of the two *Sma*I sites in the pBR323 restriction map (Fig.3).

The position and length of the colicin E1 structural gene on the pBR323 (and pBR324) map were deduced from the following facts: (a) the molecular weight of the colicin E1 protein has been reported to be approx. 56 000 daltons, thus requiring a DNA coding region of no less than $1 \cdot 10^6$ daltons (Schwartz and Helinski 1971; Meagher et al., 1976); (b) the *Sma*I site at approx. $0.87 \cdot 10^6$ daltons from the *Eco*RI site has been located in the colicin E1 structural gene (Bell et al., 1977); (c) the *Eco*RI site seems to be localized at the end of the colicin E1 structural gene (Meagher et al., 1976). The position of the colicin E1 immunity gene in pBR323 (and pBR324) has been tentatively located on the basis of data previously reported (Inselburg et al., 1977).

The *Pst*I endonuclease cleaves pBR323 into five fragments of approx. 2.38, 2.28, 0.58, 0.23 and $0.06 \cdot 10^6$ daltons (data not shown). One of these *Pst*I sites has been mapped at $0.17 \cdot 10^6$ daltons from the *Hin*cII site in the Ap^r gene (Bolivar et al., 1977a) (Fig.3). Two extra *Pst*I sites (the ones closest to the *Eco*RI and *Hin*dIII sites, Fig.3) were mapped by double *Eco*RI-*Pst*I and *Hin*dIII-*Pst*I digestions (data not shown). The positions of the remaining two *Pst*I sites have been tentatively assigned to the $0.87 \cdot 10^6$ dalton *Eco*RI-*Sma*I fragment based on *Pst*I digestions of the purified *Eco*RI-*Hin*dIII $1.1 \cdot 10^6$ dalton DNA fragment (data not shown).

A derivative of pBR323 that lacks one of the two *Sma*I sites (the one located outside the colicin E1 structural gene, approx. at 4 Mdaltons in Fig.3) was obtained by the ligation of pBR323 *Xma*I purified linears that had been treated with SI nuclease. The Ap^r, Tc^r, colicin E1 producing transformants, which occurred at a frequency of 10^{-6}/ml/μg DNA, were screened for DNA molecules which gave a linear plasmid molecule upon digestion with *Sma*I. The resulting plasmid, named pBR324, occurred at a frequency of approx. $2 \cdot 10^{-7}$/ml/μg DNA and carries only one *Sma*I (*Xma*I) site located in the colicin E1 structural gene.

Fig.3 shows the restriction map of pBR324. During the construction of pBR324 a variety of multimers with several *Sma*I sites were found at high frequency (approx. $8 \cdot 10^{-7}$/ml/μg DNA).

Construction of pBR325

P1Cm^r phage DNA was digested with the restriction enzyme *Hae*II. The resulting DNA was treated with SI nuclease and ligated to pBR322 DNA that had been previously digested with *Eco*RI and treated with S1 nuclease. The ligated mixture was used to transform *E. coli* strain RRI. The Ap^r, Tc^r, Cm^r transformants obtained were screened for plasmid DNA which gave a linear DNA molecule upon *Eco*RI digestion. The restriction pattern of one of these plasmids, pBR325, is shown in Fig.4 and the data are summarized in Fig.5.

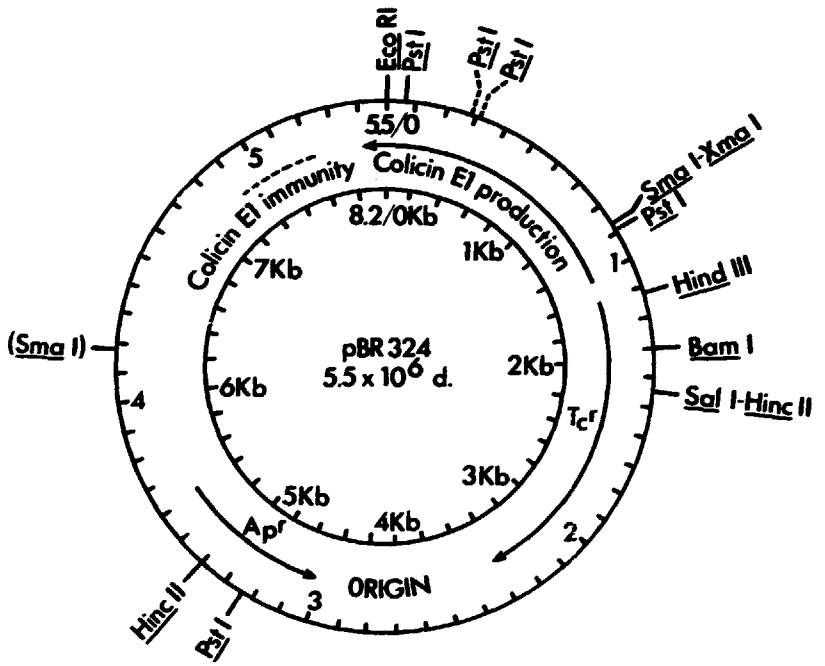

Fig.3. The circular restriction map of pBR324. The relative positions of restriction sites are drawn to scale on a circular map divided into units of $1 \cdot 10^5$ daltons and 0.2 kilobases (kb).

The estimated size of the Tc^r and colicin E1 structural genes were determined indirectly on the basis of the reported values for the size of the Tc^r associated proteins and colicin E1 protein detected in the minicell system (Meagher et al., 1976; Tait et al., 1977). The size of the Ap^r gene was determined on the basis of the nucleotide sequence of the gene in the plasmid pBR322 (G. Sutcliffe and W. Gilbert, personal communication). The position of the colicin E1 immunity gene has been tentatively localized near the colicin E1 structural gene (Inselburg et al., 1977). The direction of transcription of the Tc^r and Ap^r genes (Bolivar et al., 1977b) and the colicin E1 structural gene (Meagher et al., 1976) is indicated by arrows.

The plasmid DNA has five *Pst*I restriction sites. Three of them have been mapped unambiguously; the other two are localized in the colicin E1 structural gene and their positions have been assigned tentatively as indicated by the dotted lines. Plasmid pBR324 which has only one *Sma*I restriction site in the colicin E1 structural gene, was derived from plasmid pBR323, which has two *Sma*I restriction sites. The position of this second *Sma*I restriction site in pBR323 is indicated in the pBR324 map in parentheses; this is the only difference between pBR323 and pBR324. The position of the origin of replication of pBR324 was tentatively assigned assuming it is the same as in pBR322 (Bolivar et al., 1977b,c).

Cloning properties of pBR323 and pBR324

(1) Cloning in the Tc^r gene. The restriction sites *Hin*dIII, *Bam*HI and *Sal*I are localized in the Tc^r gene(s) carried by pBR322. Since these sites are present in the same relative positions in pBR323 and pBR324, it was assumed that they are associated with the Tc^r gene(s) present in these plasmids. To confirm this point, *N. crassa* DNA fragments produced by digestions with *Hin*dIII, *Bam*HI and *Sal*I endonucleases were cloned into their respective sites in pBR323 and pBR324. The isolated transformants carrying recombinant

plasmids, which occurred at a frequency of 10^6 to 10^{-7}/ml/μg DNA, were Ap^r, colicin E1 immune, colicin E1 producers and Tc^s (Table I).

Transformants for Tc^s carrying no recombinant DNA were obtained at a frequency of approx. 10—20 times lower.

(2) Cloning in the colicin E1 structural gene. It has been previously shown that the unique *Eco*RI site in the plasmid ColE1 is located in the colicin E1 structural gene. Since the unique *Sma*I site in pBR324 is located at $0.87 \cdot 10^6$ daltons from the *Eco*RI site, it was assumed that both of these sites were associated with the colicin E1 structural gene, as has been reported for the plasmid ColE1 (Bell et al., 1977). To confirm this point, *N. crassa* DNA fragments produced with the restriction enzymes *Eco*RI, *Sma*I and *Xma*I were cloned into their respective sites in pBR324. Cloning of *Sma*I or *Xma*I DNA fragments in pBR323 was not attempted because of the presence of an extra *Sma*I site outside the colicin E1 structural gene. Cells carrying pBR324 *Eco*RI, *Sma*I or *Xma*I recombinant molecules, which occurred at a frequency

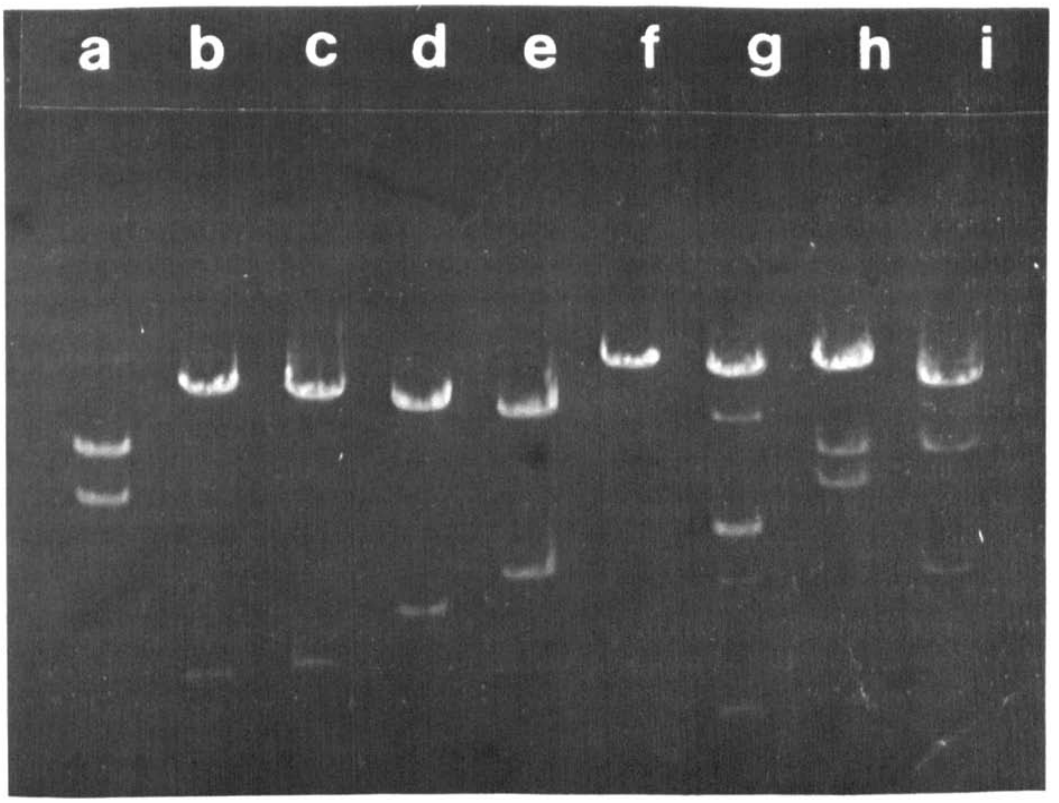

Fig.4. Agarose slab gel electrophoresis (1%) of plasmid pBR325. DNA fragments were obtained by single and double digestions using *Eco*RI, *Hin*cII, *Pst*I, *Hin*dIII, *Bam*HI and *Sal*I restriction endonucleases. Gel electrophoresis was carried out as described previously (Bolivar et al., 1977a). Molecular weight estimates are based on the *Hin*dIII generated fragments of the PM2 phage genome shown (as a partial digestion) on slot g. The restriction endonuclease digestions are as follows: Slot a, *Pst*I-*Bam*HI; Slot b, *Eco*RI-*Pst*I; Slot c, *Eco*RI-*Hin*dIII; Slot d, *Eco*RI-*Bam*HI; Slot e, *Eco*RI-*Sal*I; Slot f, *Eco*RI; Slot h, partial *Hin*cII; Slot i, partial *Hin*cII-*Eco*RI. The restriction enzymes *Hin*dIII, *Bam*HI, *Pst*I and *Sal*I cleave the pBR325 DNA molecule at a unique site (data not shown).

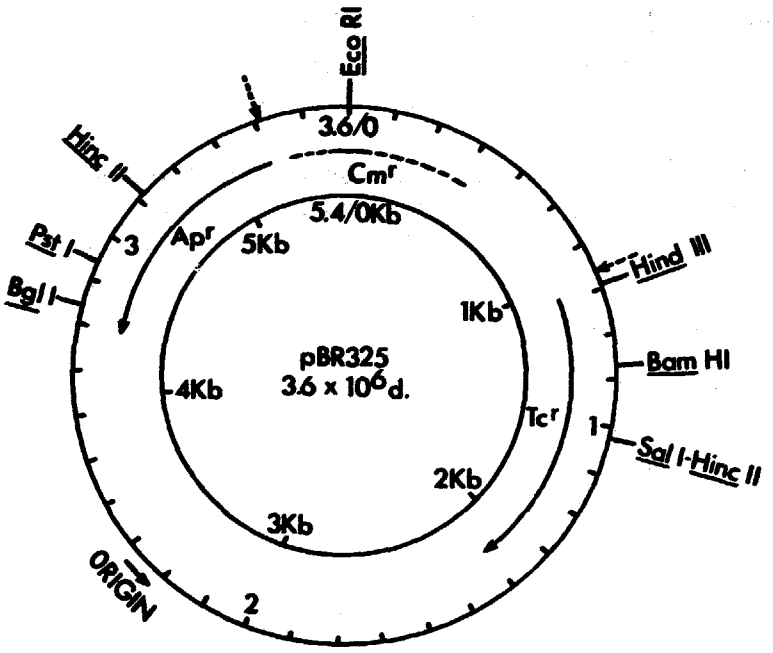

Fig.5. The circular restriction map of pBR325. The relative position of the restriction sites are drawn to scale on a circular map divided into units of $1 \cdot 10^6$ daltons. The estimated sizes of the Tc^r and Ap^r genes were determined as in the case of pBR324 (legend Fig.3). The size of the Cm^r gene was determined on the basis of reported values for the size of chloramphenicol acetyl transferase (Shaw, 1971). As in pBR324, the position of the origin of replication was assigned assuming it is the same as in pBR322 (Bolivar et al., 1977b, c). The arrows represent the insertion point in pBR322 of a 0.8 Mdalton *Hae*II fragment that carries the Cm^r gene.

of approx. $5 \cdot 10^{-7}$/ml/μg DNA, were Ap^r, Tc^r, colicin E1 immune and colicin E1 non-producers (Table I).

(3) Cloning into the Ap^r gene. The restriction endonuclease *Hinc*II, which generates blunt-ended DNA fragments, recognizes two sites in pBR323 and pBR324. One of these sites is also recognized by the restriction endonuclease *Sal*I and therefore is located in the Tc^r gene (Fig.3). The other site is assumed to be located in the Ap^r gene, as in pBR322, $0.17 \cdot 10^6$ daltons from a *Pst*I site. To demonstrate the cloning of DNA fragments into the *Hinc*II site of the Ap^r gene the following procedures were followed: DNA fragments from *N. crassa* were produced by digestion with endonuclease *Hinc*II; to preferentially cleave the *Hinc*II site in the Ap^r gene, pBR323 or pBR324 DNA were first cleaved with *Sal*I endonuclease followed by *Hinc*II digestion. These digestions generated two fragments each possessing one cohesive end (*Sal*I end) and one blunt end (*Hinc*II end in the Ap^r gene). *N. crassa* and pBR323 or pBR324 DNAs were then ligated under conditions to promote blunt-end ligation and transformed into *E. coli* strain RRI. Of the resulting Tc^r transformants, that were also Ap^s, 50% were found to carry a larger molecular weight plasmid than pBR323 or pBR324. These DNA molecules contained

extra DNA fragments cloned at the *Hinc*II site in the Ap^r gene (Table I).

Cloning properties of pBR325

(1) Cloning in the Tc^r and Ap^r genes. Plasmid pBR325 carries unique restriction sites for *Hin*dIII, *Bam*HI, *Sal*I and *Pst*I. As in pBR322, cloning in the first three sites inactivates the Tc^r gene(s) while cloning in the *Pst*I site inactivates the Ap^r gene (Table I). Cloning into the *Hinc*II site of the Ap^r gene was achieved using the same strategy described for pBR322 (Bolivar et al., 1977b), pBR323 and pBR324. These transformations yield recombinant DNA molecules at frequencies similar to those reported for pBR322.

(2) Cloning of BglII generated fragments in the BamHI site of pBR325. *Bgl*II generated DNA fragments from *N. crassa* were cloned into the *Bam*HI

TABLE I

MOLECULAR CLONING OF VARIOUS DNA FRAGMENTS IN pBR324 AND pBR325

The molecular weight (Md 10^{-6}) under each restriction site represents DNA fragments of independent clones isolated in this laboratory.

DNA source	Cloning in pBR324							
	Restriction endonuclease substrate site used for cloning							
	*Eco*RI	*Hin*dIII	*Bam*HI	*Sal*I	*Xam*I	*Hinc*II (in the Ap^r gene)	*Sma*I-*Xma*I	
N. crassa	3.2	4.6	6.1	2.1	2.4	3.4	2.1	4.1
	1.5	1.2	2.1	1.2	0.5	2.1	1.2	3.8
	0.4							
E. coli Strain GM48			3.4[a]					
			2.1[a]					
			0.9[b]					
			0.5[b]					
	Cloning in pBR325							
	Restriction endonuclease substrate site used for cloning							
	*Eco*RI	*Hin*dIII	*Bam*HI	*Sal*I	*Xam*I	*Pst*I	*Hinc*II (in the Ap^r gene)	
N. crassa	2.1	2.1	3.4	4.5	3.2	4.1	2.1	
	1.8	0.6	2.1	0.5	1.5	3.2	1.8	
	0.5							
E. coli Strain GM48			3.1[a]					
			1.1[a]					
			1.0[b]					
			0.8[b]					

[a] *Bgl*II-generated DNA fragments cloned in the *Bam*HI site. The size of the cloned fragments was estimated by comparing the linear *Eco*RI recombinant molecules with the six *Eco*RI generated fragments (data not shown).

[b] *Dpn*II-generated DNA fragments cloned in the *Bam*HI site.

site of pBR322 and pBR325. Cloning resulted in the inactivation of the Tc^r mechanism of the plasmid (Table I). Transformants carrying a larger molecular weight plasmid occurred at a frequency of $2 \cdot 10^{-7}$/ml/μg DNA.

(3) Cloning of DpnII generated fragments in the BamHI site of pBR325. *E. coli* strain GM48 DNA was digested with the restriction enzyme *Dpn*II and the resulting DNA was ligated to pBR325 DNA previously digested with *Bam*HI. After ligation the DNA mixture was used for transforming *E. coli* strain RRI. Transformants for Ap^r, Tc^s, Cm^r occurred at a frequency of approximately $5 \cdot 10^{-7}$/ml/μg DNA. Certain cloned *Dpn*II fragments (less than 10%) were recovered as *Bam*HI fragments (data not shown).

(4) Cloning in the Cm^r gene. *Eco*RI generated DNA fragments from *N. crassa* were ligated with linear *Eco*RI digested pBR325. Upon transformation the resulting recombinant plasmids, occurring at a frequency of approx. $8 \cdot 10^{-7}$/ml/μg DNA, gave Ap^r, Tc^r, Cm^s, phenotypic cotransformants thus, analogous to the case of R65 (Chang and Cohen, 1977), a unique *Eco*RI site is located in the Cm^r gene (Table I). Transformants for Tc^r, Ap^r, Cm^s apparently carrying no recombinant DNA molecules were found at a frequency of approx. $8 \cdot 10^{-8}$/ml/μg DNA.

DISCUSSION

The plasmids pMB9, pBR313 and pBR322 have been widely used in the cloning and expression of interesting natural and synthetic DNA fragments. Most of the molecular cloning in these vectors has been achieved by taking advantage of the presence of unique endonuclease restriction sites in the antibiotic resistant genes carried on the plasmids, thus allowing for an easy selection of recombinant DNA molecules.

The position of a unique *Eco*RI restriction site outside any structural gene in these plasmids has not encouraged the molecular cloning of *Eco*RI generated fragments because of the lack of an inactivated marker that would allow recognition of cells harboring recombinant plasmid molecules. Moreover, it is well known that many natural structural genes do not contain *Eco*RI sites in their sequences (O'Malley et al., 1976; Seeburg et al., 1977; Ullrich et al., 1977) and yet many interesting DNA fragments can be obtained after *Eco*RI digestion.

I therefore decided, based on the large quantities of pure and active *Eco*RI that can be obtained after an easy purification scheme (Greene et al., 1978) and the commercial availability of synthetic oligonucleotides carrying the *Eco*RI recognition sequence (Scheller et al., 1977), to construct a plasmid cloning vector which would permit easy selection of *Eco*RI recombinant molecules.

Two pBR322 derivatives were constructed, each carrying an extra structural gene with a unique *Eco*RI site. The incoming structural gene was cloned into the *Eco*RI site of pBR322 in such a way that the ligation of the *Eco*RI-cut linear pBR322 plasmid DNA with the heterologous restriction fragment re-

sulted in the destruction of the *Eco*RI termini provided by the vector. This objective was achieved in two different ways depending upon the type of termini provided by the heterologous fragment carrying the *Eco*RI site.

Two different structural genes were selected for insertion into pBR322, the colicin E1 structural gene and the gene coding for chloramphenicol resistance. The colicin E1 structural gene along with the immunity gene were obtained from plasmid pMBI in a *Hin*cII blunt-ended generated fragment of about $2.8 \cdot 10^6$ daltons. This blunt-ended fragment was ligated to the linear *Eco*RI pBR322 DNA molecule whose cohesive *Eco*RI termini were previously filled by T4 DNA polymerase to form a blunt-ended fragment. When these two blunt-ended fragments were ligated, neither the *Hin*cII nor the *Eco*RI sites were recovered. From this experiment we obtained plasmid pBR323. The Cm^r gene can be obtained from the phage P1Cm^r in a *Hae*II restriction fragment of approx. $0.8 \cdot 10^6$ daltons (unpublished observations). After the digestion of P1Cm^r phage DNA with this restriction enzyme, the DNA was treated with SI nuclease to eliminate the 3′ OH protruding *Hae*II termini. This DNA was then ligated to SI nuclease-treated pBR322 *Eco*RI generated linear molecules to form plasmid pBR325. Plasmid pBR324 a derivative of plasmid pBR323 that carries a unique *Sma*I (or *Xma* I) site, was constructed in order to obtain a *Sma*I (or *Xma*I) cloning vector. In confirmation of the results of Bell et al. (1977), it was proved that the cloning of either *Sma*I blunt-end generated fragments or *Xma*I cohesive-ended DNA fragments inactivates the colicin E1 structural gene in plasmid pBR324. It was possible to recover these fragments after digestion with either of the two enzymes.

The absence of *Pst*I sites in the Cm^r gene of pBR325 still permits the use of the unique *Pst*I site in the Ap^r gene for cloning using the deoxypolymeric extension technique which allows for the recovery of a "C" tailed cloned fragment such as a *Pst*I fragment (Bolivar et al., 1977b). However, in pBR323 and pBR324, the presence of at least three (possibly four) *Pst*I sites in the colicin E1 structural gene does not allow for *Pst*I cloning.

We have also demonstrated that cloning *Eco*RI generated DNA fragments in the *Eco*RI site of pBR325 inactivates the Cm^r gene thus allowing for an easy selection of recombinant DNA molecules. The same is true for the cloning of *Eco*RI fragments in pBR324 (and pBR323); cells carrying recombinant DNA molecules can be easily detected due to their inability to produce colicin E1 as compared to cells carrying only the vector. Furthermore, the possible location of the *Eco*RI site close to the nucleotide sequence coding for the —COOH terminal of the colicin E1 structural gene (Meagher et al., 1976) suggests the possibility of using this gene as an alternative to the β galactosidase system for the protection of small peptides whose synthetic or natural coding DNA sequence(s) are inserted at the *Eco*RI site (Itakura et al., 1977).

As expected, cloning *Hin*dIII, *Bam*HI and *Sal*I generated DNA fragments in pBR324 and pBR325 inactivates the Tc^r gene(s). The same happens when *Xam*I (an isochizomer of *Sal*I) blunt-end generated fragments are cloned in the Tc^r gene (Table I). In all four cases the cloned DNA pieces can be re-

covered after redigestion of the recombinant DNA molecule with the enzyme.

It is possible to clone *Bgl*II-generated fragments in pBR322, pBR324 and pBR325 and inactivate the Tc^r gene because the *Bgl*II and *Bam*HI restriction endonucleases share the same cohesive termini GATC. However, it is not possible to recover the cloned fragments as a *Bgl*II or *Bam*HI fragment. Nevertheless the fragment can be recovered by digestion with the restriction enzyme *Dpn*II (if no other *Dpn*II site is present in the cloned fragment).

*Dpn*II fragments can be cloned in the *Bam*HI site of these plasmids and a small fraction of them can be recovered after *Bam*HI digestion.

In summary, the construction of these two plasmids, pBR324 and pBR325, now permits the molecular cloning and easy selection of *Eco*RI, *Bam*HI, *Bgl*II, *Dpn*II, *Hin*dIII, *Sal*I, (*Xam*I), *Pst*I, *Hin*cII and *Sam*I (*Xma*I) recombinant DNA molecules. Experiments are now being performed in order to apply for the approval of these vectors in *E. coli* strain χ1776 as EK2 systems.

ACKNOWLEDGEMENTS

I wish to thank Heather Stieglitz and Fernando Bastarrachea for critically reading the manuscript.

This research was supported by a Grant from P.N.C.B. Consejo Nacional de Ciencia y Tecnología, México.

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Communicated by D.R. Helinski.