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MINIREVIEW

MOLECULAR CLONING VECTORS DERIVED FROM THE CoLE1 TYPE PLASMID pMB1

Francisco Bolívar

Departamento de Biología Molecular, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Apdo. postal 70228, México 20, D. F.

Summary

In vitro recombination via restriction endonucleases and *in vivo* genetic translocation of an ampicillin resistance gene, have been utilized for the construction of a series of cloning vehicles. These vectors have been derived by combining the essential replication properties of plasmid pMB1, a CoLE1 type plasmid, with one, two or three resistance markers (tetracycline, ampicillin, chloramphenicol and colicin E1 production). During the construction of these vectors, the position of restriction sites used for cloning DNA fragments, relative to the antibiotic resistance genes and the origin of DNA replication, have been determined. The use of the cloning vectors pMB9, pBR313, pBR322, pBR324, pBR325 permits the molecular cloning and easy selection of *Eco* RI, *Bam* HI, *Bgl* II, *Dpn* II, *Hind* III, *Sal* I, *Xho* I, *Xam* I, *Taq* I, *Pst* I, *Hinc* II, *Pvu* I, *Sma* I, *Xma* I, *Bal* I, *Hpa* I, *Ava* I, *Pvu* II, *Hae* III, *Alu* I, *Hpa* II, *Eco* RII and virtually any blunt-ended generated DNA fragment.

There are several essential steps in *in vitro* recombinant DNA technology required for the insertion of DNA fragments from any source into either viral or plasmid replicons. Generally speaking, these steps involve the specific cleavage of the desired DNA with restriction endonucleases (1), the covalent joining of DNA fragments to the cloning vehicle (2), and the transformation of a suitable bacterial host strain, with the recombinant DNA molecules (3). The recovery of bacterial clones containing replicating recombinant plasmids can be readily obtained with this procedure. It is now clear that one of the most critical component of this technology is the cloning vehicle (4)

Bacterial plasmids have been used as molecular cloning vehicles for the stable maintenance of DNA in bacteria (3, 5, 6). One can itemize the essential features required for a useful cloning vector as follows. First of all, the plasmid should be stably maintained in the host bacterial cell. Second, it should be a non self transmissible genetic element. Third, the plasmid must be purified easily and in sufficient quantities for experimentation. Fourth, one must have two or more genetic markers associated with the plas-

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mid which provides for the screening or selection of transformed cells carrying recombinant molecules. Fifth, the vehicle should have the capacity of joining with foreign DNA of a broad size range. In addition to these properties the more recently developed plasmid cloning vehicles have one or more of the following properties:

a) Multicopy number
b) Amplification of the DNA upon incubation of the carrying cells in the presence of chloramphenicol or other protein inhibitors.
c) Reduced in size.
d) The presence of unique restriction sites preferentially located in the structural genes for the genetic markers.
e) The knowledge of the complete DNA nucleotide sequence of the vehicle.
f) The presence of prokaryotic regulatory genes that promote high level expression of introduced foreign DNA in bacteria.

These various properties of plasmid DNA molecules are directed toward increasing their efficacy and safety as cloning vehicles. This review is concerned with the design, construction and characteristics of a collection of plasmid vectors derived from the CoLE1 type plasmid pMB1 (7), that have been widely used in molecular cloning of DNA.

Construction and characterization of pMB8 and pMB9.

By using *in vivo* genetic translocation of resistance genes and *in vitro* recombination techniques, naturally occurring plasmids can be modified to facilitate the manipulation of cloned DNA and to provide a high level of biological containment of recombinant plasmids.

The construction and characterization of a series of pMB1 derivatives have been reported (7, 8, 9). From one of these plasmids, named pMB3, that carries the *Eco* RI nuclease and methylase genes, a small derivative was constructed after digestion of pMB3 DNA with the *Eco* RI* activity of the *Eco* RI endonuclease (6). This plasmid, named pMB8, is amplifiable by the use of chloramphenicol, has a molecular weight of 1.75×10^6 daltons (d), and carries the colicin E1 immunity gene as well as unique *Eco* RI and *Sma* I sites (Fig. I). Although this small plasmid has some advantage as a cloning vector (small size and low backgroung of protein synthesis in minicells), it does not have a good selective marker and properly localized unique restriction sites (6, 9). Therefore plasmid pMB9 was constructed in order to overcome some of the disadvantages of pMB8 (Fig. 1).

Plasmid pMB9 carries the replicating elements of pMB8 and the tetracycline resistance (Tc^r) gene from pSC101 (3). This plasmid was constructed by ligating the products of an *Eco* RI* endonuclease digest of pSC101 DNA with an *Eco* RI endonuclease digest of pMB8 (6, 9). pMB9 has a molecular weight of 3.5×10^6 d. and one substrate site for each of the following restriction endonucleases: *Eco* RI *Hind* III, *Sal* I and *Bam* HI. The relative position of these sites (Figs. 1-2) was determined by gel electrophoresis of double and triple digestions of plasmid DNA with various restriction enzymes. Since pMB8 has no *Hind* III, *Bam* HI or *Sal* I sites, these sites should be associated with the pSC101 DNA fragment introduced in pMB9. This assumption is supported by the fact that these three sites are - - present in pSC101 in the same relative positions and that the molecular cloning of DNA into the *Hind* III, *Bam* HI and *Sal* I sites alters the Tc^r mechanism (6, 9, 10). The fact that DNA inserted into the *Eco* RI site of pMB9 does not alter the expression of the Tc^r mechanism, indicates that this site lies outside the Tc^r gene. However, cloning into the *Eco* RI site of pSC101 has been found to affect the level and inducibility of Tc^r (11).

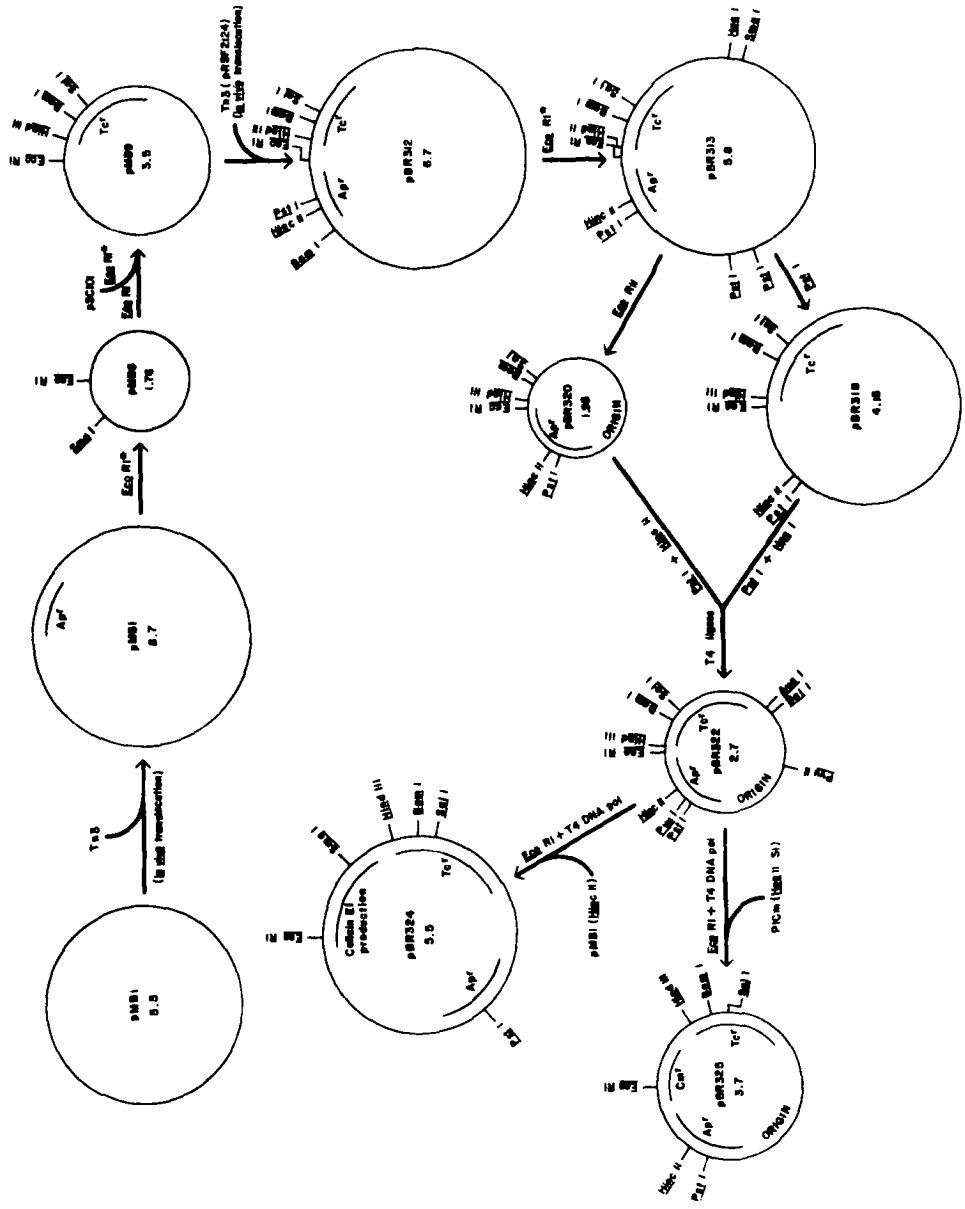

FIG. I.

Diagrammatic representation of the construction of pMB8 pMB9, pBR313, pBR322, pBR324 and pBR325. All these cloning vectors have been derived from the parental plasmid pMB1 via in vitro DNA manipulations and in vivo translocations. For detailed explanation see the text.

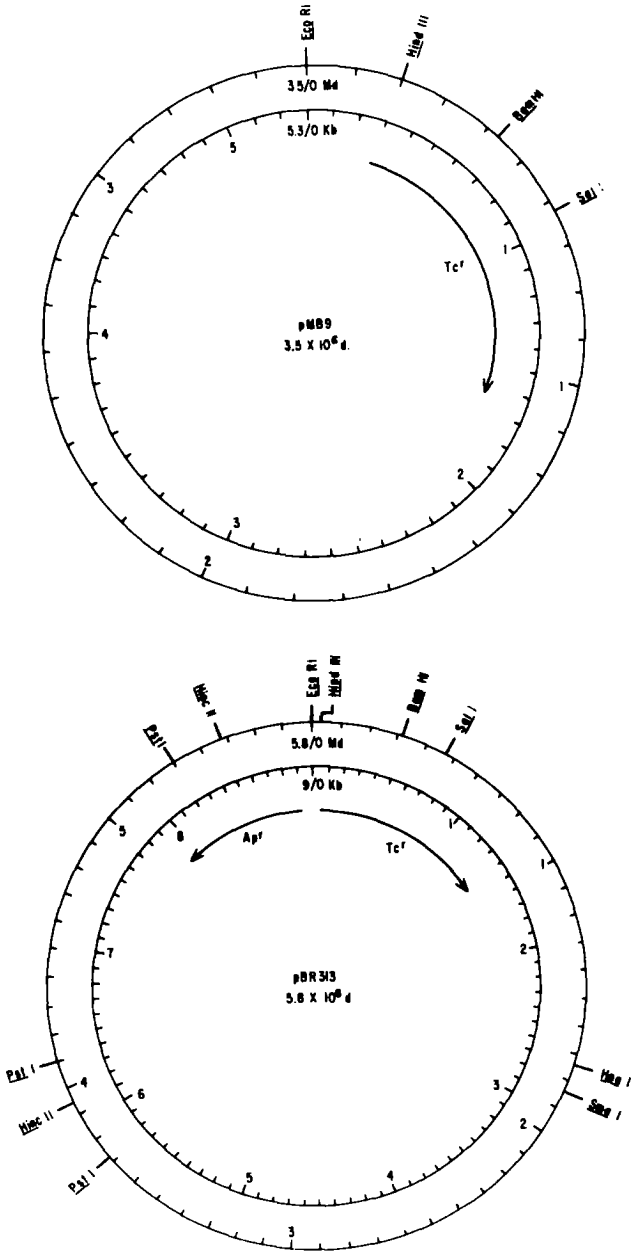

FIG. 2

The circular restriction maps of pMB9 and pBR313. The relative position of restriction sites are drawn to scale on circular maps divided into units of 1 x 10^5 daltons and 0.1 kilobases (kb).

Although Eco RI-generated recombinant plasmids of pMB9 can be selected by virtue of their resistance to tetracycline, transformants with recombinant plasmids constructed by insertion of DNA fragments generated by Hind III, Bam HI and Sal I digestion and incorporated into the respective restriction sites can only be selected by colicin E1 immunity. Since the action of colicin E1 is extremely dependent on the physiological state of the cell and is accompanied by a high frequency of spontaneous mutations to colicin tolerance (12), it was decided to introduce another selective marker into pMB9. This was accomplished by the genetic translocation to pMB9 of an ampicillin resistance marker (Ap^r) present in the translocatable element Tn3 from pRSF2124 (13, 14).

Construction of Ap^r-Tc^r derivatives of pMB9

Translocation of Tn3 from pRSF2124 to pMB9 was accomplished by cotransforming E. coli with supercoiled pMB9 and pRSF2124 DNAs. Ap^r-Tc^r transformants were screened for plasmid DNA which gave a linear plasmid molecule upon digestion with Eco RI. Some of these Eco RI-generated linear molecules had molecular weights of 6.7×10^6 d. which corresponds to the sum of the molecular weights of the Tn3 (3.2×10^6 d.) and pMB9. These plasmid molecules were shown to confer resistance to Ap and Tc when transformed back into E. coli.

The presence of a single asymmetrically located Bam HI site about 1×10^6 d. from the end of the Tn3 (13), made it possible to localize the various Tn3 insertion sites in pMB9. One Ap^r - Tc^r derivative of pMB9 designated pBR312, that carries a translocation insertion near the Eco RI site, was chosen for further study (Fig. I). This plasmid has two Bam HI sites, one of which is in the translocated segment. In order to remove this site and reduce the overall size of the plasmid, pBR312 plasmid DNA was subjected to Eco RI* digestion and then used to transform E. coli. Ap^r-Tc^r transformants were selected and their plasmids were screened for the loss of the Bam HI site in the translocated fragment by digestion with Bam HI restriction endonuclease. Several plasmids with only one Bam HI site were recovered and each plasmid had lost about 1.0 to 1.5 megadaltons of DNA. Interestingly enough, the Eco RI site was lost in one case, pBR316, and in other two cases, pBR313 and pBR314, the position of the Eco RI site with respect to the Hind III site had changed (9). The smallest generated plasmid, pBR313, was characterized (Fig. 2). The relative positions and distances of the Eco RI, Hind III, Bam HI and Sal I sites in pBR313 are indistinguishable from those in pSC101. Presumably in the construction of pMB9, the Eco RI site near the Hind III site was rejoined with an Eco RI* fragment to produce an Eco RI* site. Therefore in the construction of pBR313, the Eco RI site near the Hind III site was restored. The relative position of various restriction sites in pBR313 have been determined and a restriction map is shown in figure 2.

Construction of pBR322.

As can be seen in figures 1 and 2, pBR313 has one of its three Pst I sites located in the Ap^r gene (6, 9). 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 (15). An Ap^s-Tc^r-Col^{imm} plasmid, pBR318, containing one Pst I site was obtained by transforming E. coli 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 d. Pst I fragments present in pBR313 (Fig. 1). Another pBR313 derivative, pBR320, an Ap^r-Tc^s-Col^s plasmid with a molecular weight of 1.95×10^6 daltons, was obtained by transforming E. coli with unligated Eco RII fragments of pBR313 and selecting for Ap^r transformants. Ap^r-Tc^s clones were examined, and

one was found to carry this plasmid, pBR320, containing only one Pst I site.

An in vitro recombination experiment using pBR318 and pBR320 was designed to restore the Ap^r-Tc^r markers in a single low molecular weight relaxed plasmid containg one Pst I substrate site (15). 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 x 10^6 d.; the smaller DNA fragment carried the Tc^r gene(s) and part of the Ap^r gene as shown in figure 1. The plasmid pBR320 was cleaved with the restriction enzymes Pst I and Hinc II to yield three fragments of DNA. The largest fragment, 1.15 x 10^6 d., carries the origin of replication (16) and the remaining portion of the Ap^r gene not present in the 1.95 x 10^6 d. fragment of pBR318. The digested DNAs were mixed, ligated in vitro, and transformed into E. coli. Since neither pBR320 nor the 1.95 x 10^6 d. fragment of pBR318 carry the colicin El immunity gene, transformants were selected for Ap^r, Tc^r and then screened for sensitivity to colicin El, This transformation yielded numerous Ap^r-Tc^r-Col^s clones which carried a plasmid (e.g. pBR321) with a molecular weight of 3.1 x 10^6 d. As expected, this plasmid resulted from the addition of the 1.95 x 10^6 d. Hpa I - Pst I fragment of pBR318 and the 1.15 x 10^6 d. Pst I - Hinc II DNA piece from pBR320.

From this transformation a smaller, 2.7 x 10^6 d., Ap^r-Tc^r-Col^s plasmid was also obtained. This plasmid, possibly the result of an in vivo recombination event near unligated termini, was missing approximately 0.4 x 10^6 d. of DNA from a region of pBR321, not associated with Ap^r, Tc^r or DNA replication (15). Because of its lower molecular weight compared with pBR321, pBR322 was chosen for further characterization.

Plasmid pBR322 is a very versatile cloning vehicle; this plasmid has 4361 base pairs (bp) (17), and as pointed out contains two selective markers Ap^r and Tc^r. Neither of these genes is on transposable elements and neither can be transposed from the cloning vehicle to another plasmid. pBR322 has one Pst I and one Pvu I sites located in the Ap^r gene, one Bam HI, one Hind III, one Sal I and one Sph I (Y. Fuchs and F. Bolivar, submitted for publication), sites all located in the Tc^r gene. It has one Eco RI site which lies between the promoters of the Ap^r and Tc^r genes and also carries unique Pvu II, Bal I and Ava I sites close the 3' end of the Tc^r gene(s). The relative positions of these restriction endonuclease substrate sites were originally determined by double and triple digestions in pBR322 DNA (15). However, the complete nucleotide sequence of pBR322 was recently determined by G. Sutcliffe (17) and this allowed for the precise localization of all restriction sites present in the DNA molecule, as shown in figure 3.

Cloning DNA in pBR322

The advantage of having two selective markers becomes obvious in view of the locations of the Pst I, Pvu I, Sal I, Bam HI, Sph I and Hind III restriction sites. Fragments of DNA generated by the Pst I endonuclease, for example, when cloned in the pBR322 plasmid result in the loss of Ap^r (4, 15). Tc^r transformants carrying recombinant DNA molecules derived from such an experiment can be identified as Ap^s. In addition, the position of the cleavages made by the Pst I endonuclease leave protruding 3' -OH single strands of DNA which are excellent primers for terminal transferase addition of deoxyribonucleotides. If deoxyguanosine triphosphate is used as a substrate for "tailing" of the Pst I digested plasmid, and deoxycytidine triphosphate is used for "tailing" DNA fragments, then the Pst I restriction site can be restored upon reannealing of the two DNA components (15). For example, this approach has been successful for cloning cDNA, (18) and other Pst I generated

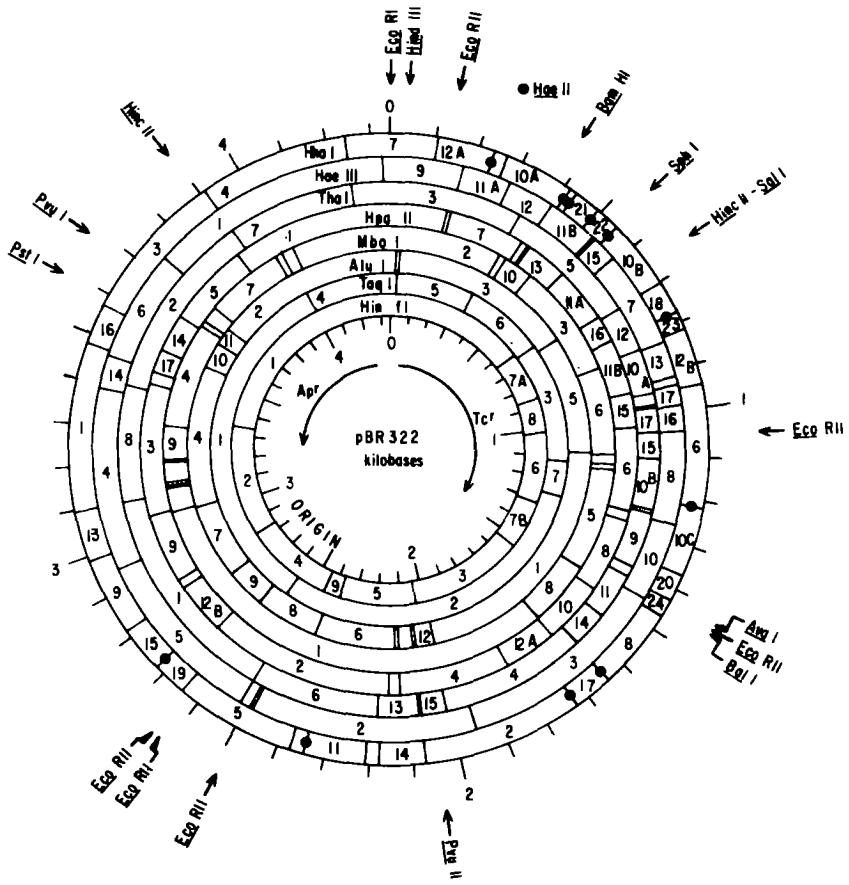

FIG. 3

The circular restriction map of pBR322. The precise location of all restriction sites in pBR322 DNA has been determined by the knowledge of the complete nucleotide sequence of this DNA molecule (17).

fragments (19), and the cloned DNAs can be precisely removed with Pst I endonuclease treatment of the recombinat plasmid. Moreover, a simple method has been described that allows for detection of Ap^r and Ap^s clones by the use of the dye N-phenyl-1-naphthylamine-azo-o-carboxybenzene (20). The main advantage of the tailing method over direct Pst I cloning is that after tailing the vector and DNA fragments they cannot recircularize with themselves, and only recombinant DNA (90%) molecules can be recovered after transformation.

Cloning of DNA fragments generated by Hind III, Sal I, or Bam HI restriction endonucleases can likewise be detected by screening Ap^r transformants for Tc^s. More importantly, Tc^s transformants can be quantitively selected by inhibiting their growth with tetracycline and killing the actively growing Tc^r (nonrecombinant plasmid) transformants with D-cycloserine (6). The other advantage of this cloning vehicle and derivatives is the possibility of using combinations of two different restriction sites for cloning fragments of DNA. In particular, this can be used for subcloning fragments of DNA for sequence analysis.

Construction of pBR324 and pBR325.

Although pBR322 has many advantages as a cloning vector, it also has some disadvantages. As mentioned before, the unique Eco RI site in pBR322 is located 21 bp. from the Hind III site, which has been reported to be localized in the Tc^r promoter (8, 15, 21), and 207 bp. from the putative AUG (f-met) of the Ap^r gene (15, 17). Therefore cloning into the Eco RI site of pBR322 does not affect the antibiotic resistant phenotype of cells carrying Eco RI recombinant molecules.

In order to overcome this disadvantage, two pBR322 derivatives were constructed, each carrying an extra structural gene with a unique Eco RI site (Figs. 3 and 4). The incoming structural gene was cloned into the Eco RI site of pBR322 in such a way that the ligation of the Eco RI digested linear pBR322 plasmid DNA with the heterologous restriction fragment resulted in the destruction of the Eco RI termini provided by the vector. This objetive was achieved in two different ways depending upon the type of termini provided by the heterologous DNA fragment carrying the Eco RI site (22, 23). Two different structural genes were selected for insertion into pBR322; the colicin E1 structural gene and a gene coding for chloramphenicol resistance. The colicin E1 structural gene along with the immunity gene were obtained from plasmid pMB1 in a Hinc II blunt-ended generated fragment of about 2.8×10^6 d. This blunt-ended fragment was ligated to a linear Eco RI pBR322 DNA molecule whose cohensive 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 Hinc II nor the Eco RI site were recovered. From this experiments we obtained plasmid pBR323. 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 Xam I) cloning vector. It was proved that the cloning of either Sma I blunt-ended generated fragments or Xma I cohesive ended DNA fragments inactivates the colicin E1 structural gene in plasmid pBR324. The Cm^r gene was obtained from the phage P1Cm^r in a Hae II restriction fragment of approx. 1×10^6 d. After digestion of P1Cm^r phage DNA with this restriction enzyme, the DNA was treated with S1 nuclease to eliminate to 3' OH protruding Hae II termini. This DNA was then ligated to S1 nuclease treated pBR322 Eco RI generated linear molecules to form plasmid pBR325 (23).

Cloning Eco RI generated DNA fragments into 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. 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 extention technique. 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.

As expected, cloning Hind III, 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) generated fragments are cloned in the Tc^r gene. In all four cases the cloned DNA pieces can be recovered after redigestion of the recombinant DNA molecule with the respective enzyme (23).

It is possible to clone Bgl II-generated fragments in pBR322, pBR324 and pBR325 and inactivate the Tc^r gene because 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 fragments. Nevertheless, the fragments 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

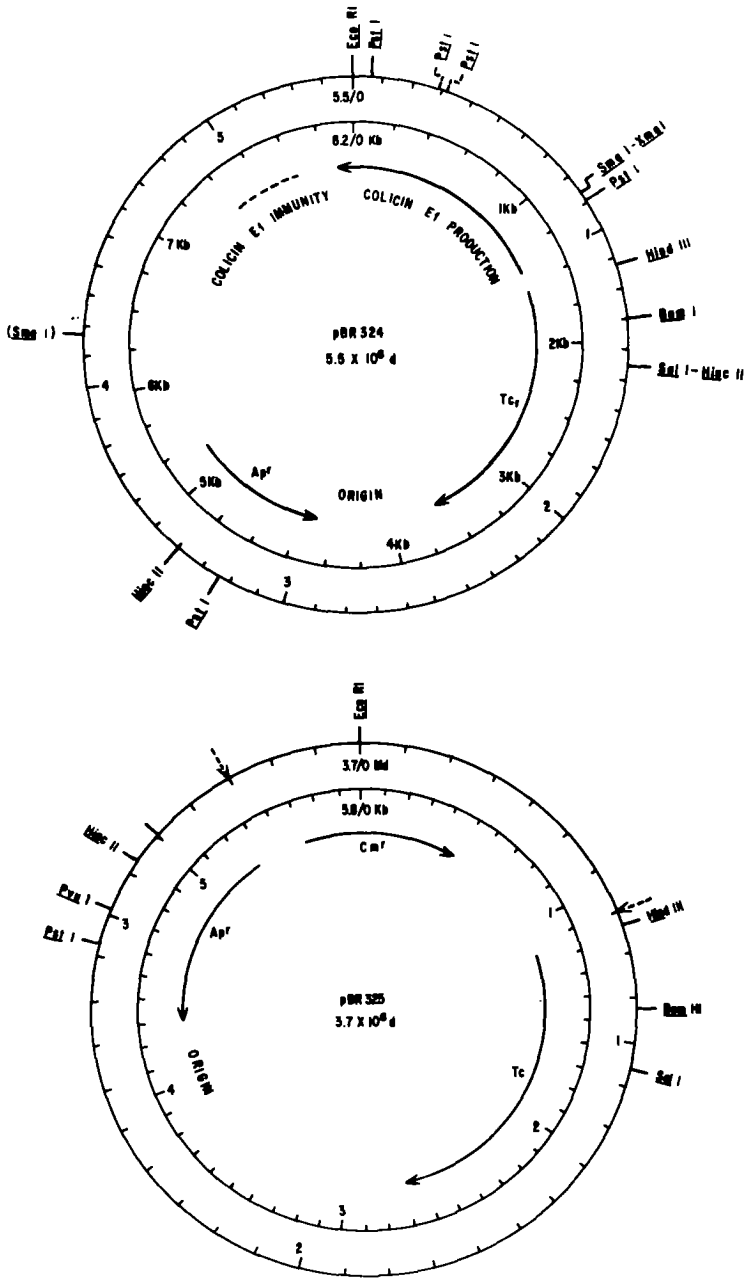

FIG. 4.

The circular restriction maps of pBR324 and pBR325. The relative positions of restriction sites are drawn to scale on circular maps divided into units of 1 x 10^5 daltons and 0.1 kilobases (kb). The arrows indicate the position of the insert into plasmid pBR322. For detailed explanation see the text.

can also be cloned in the Bam HI site of these plasmids and some of them can be recovered after Bam HI digestion. It is also possible to clone Xho I generated DNA fragments in these plasmids because Xho I and Sal I endonucleases share the same cohesive termini TCGA. Also, as in the Bgl II - Bam HI case, it is not - possible to recover the cloned fragments as Sal I or Xho I, but the fragments can be recovered by digestion with the endonuclease Taq I (if no other Taq I site is present the cloned DNA segment).

Hae III and Alu I DNA generated fragments can be cloned in pBR322 or pBR325 and recovered as Eco RI, Bam HI or Sal I fragments. This is possible by digesting the vector with one of the three enzymes, "filling up" the cohesive ends with T4 DNA polymerase and blunt-end ligate the DNA fragments. The same is true for Hpa II and Eco RII produced DNA segments; the only difference is that Hpa II and Eco RII generated fragments have to be treated with T4 DNA polymerase prior to blunt-end ligation.

In summary, the construction of these plasmids pMB9, pBR313, pBR322, pBR324 and pBR325, now permits the molecular cloning and easy selection of Eco RI, Bam HI, Bgl II, Dpn II, Hind III, Sal I, (Xam I), Xho I, Pst I, Hinc II, Pvu I, Sma I (Xma I), Hae III, Alu I, Hpa II and Eco RII recombinat DNA molecules. Moreover any kind of blunt-ended DNA fragment can be clone and easily recovered from these vectors by the use of synthetic oligonucleotides ("linkers") (24).

Derivatives of pBR322 that allow the expression of foreign DNA.

The cloning vehicle pBR322 has been used for the cloning of a wide variety of DNA fragments generated from different sources. In certain instances, it has been possible to express cloned eukaryotic DNA in E. coli when cloned in - pBR322. This has been achived by different groups taking advantage of the fact that the prometer of the lactamase (Ap^r) gene in pBR322 is a very strong one. By cloning proinsulin cDNA in the Pst I site of the Ap^r gene, Villa Kamaroff et al (18) demonstrated that the E. coli strain carrying this recombinant plasmid was able to produce a polypeptide containing the proinsulin aminoacid sequence. The same strategy was used by Seeburg et al. for the expression of the growth hormone (25). An alternative route for obtaining expression of cloned foreign DNA in E. coli was followed by Rao and Rogers (26). They constructed a derivative of pBR322 that carries the P_L promoter from bacteriophage λ .Using this plasmid, they were able to overproduce E. coli exonuclease III. A similar - strategy was employed by Itakura et al. (27) and Goeddel et al. (28) for the produccion of the hormones somatostatin and insulin in E. coli; however instead of using the P_L promoter they constructed pBR322 derivatives carrying regulatory regions and part of the galactosidase gene from the lac operon.

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