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SHORT COMMUNICATION

Molecular Cloning in Plasmid pBR322 giving Altered Expression of the Tetracycline Resistance Gene

By MARCEL BASTIN

Département de Microbiologie, Centre Hospitalier Universitaire de Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada

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The two *Hin*dIII fragments of polyoma virus DNA were cloned in the *Hin*dIII site of plasmid pBR322, a site located in the RNA polymerase promoter involved in the expression of tetracycline resistance. Although insertion of foreign DNA into this site did not always result in the complete loss of tetracycline resistance, *Escherichia coli* K12 strain χ1776 harbouring recombinant plasmids exhibited reduced growth properties in liquid culture with tetracycline and could easily be differentiated from bacteria transformed by non-recombinant plasmids. The formation of plasmid multimers increased the resistance to tetracycline at the level of the induction period, presumably as a result of a gene dosage effect.

INTRODUCTION

The usefulness of plasmid cloning vehicles is determined primarily by the ease with which recombinant and non-recombinant plasmids can be differentiated. Most of the methods for identification of clones carrying the desired chimeric plasmid involve the use of radioactively labelled DNA or RNA probes (Grunstein & Hogness, 1975) or the inactivation of genetic markers such as antibiotic resistance genes (Helling & Lomax, 1978). A feature of plasmids of the pBR series that renders them extremely valuable is the possibility of insertional inactivation in several antibiotic resistance genes (Bolivar *et al.*, 1977). In pBR322, currently the most useful vector, DNA fragments cloned in the *Pst*I site inactivate the ampicillin (Ap) resistance gene (*amp*). Insertions in the *Bam*HI or *Sal*I sites inactivate the tetracycline (Tc) resistance gene (*tet*) (Bolivar *et al.*, 1977). Alterations in the region of the *Hin*dIII and *Eco*RI sites result only in a decrease in the level of Tc resistance because these sites lie in or near the RNA polymerase promoter involved in the expression of the Tc resistance mechanism (Rodriguez *et al.*, 1977). As not all insertions in this region result in Tc sensitivity, cloning of DNA fragments in the *Hin*dIII site of pBR322 is not usually detected by screening transformants for a Tc-sensitive phenotype.

In this work, we used the *Hin*dIII site of pBR322 to clone the two *Hin*dIII fragments of polyoma (Py) virus DNA. We found that under appropriate conditions of bacterial growth, clones harbouring a plasmid with an altered *tet* gene had a distinct phenotype which made it possible to screen for transformants with insertions of foreign DNA fragments in the *Hin*dIII site of pBR322. We showed that the difficulty of growing bacteria with partially inactivated *tet* could be circumvented by cloning plasmid multimers in a *recA*$^-$ host so as to increase the level of resistance to the antibiotic.

METHODS

Preparation of DNA. Mouse 3T6 cells were infected, at a multiplicity of about 10 plaque-forming units per cell, with the A2 large plaque or the P16 small plaque variants of Py virus, and supercoiled Py DNA was isolated by the Hirt procedure (Hirt, 1967) followed by equilibrium centrifugation in CsCl–ethidium bromide solution (Bauer & Vinograd, 1968).

Plasmid DNA was isolated from *E. coli* K12 strain χ1776 according to Curtiss *et al.* (1977). The solution was treated with phenol in the presence of 0·1% (w/v) sodium dodecyl sulphate (SDS), extracted with a mixture of chloroform/isoamyl alcohol (24 : 1, v/v), and the DNA was precipitated by addition of salt (0·1 M-NaCl) and 2 vol. ethanol. The samples were purified further by Sepharose filtration to remove SDS and/or RNA whenever they were to be cleaved with restriction enzymes or used in transformation experiments. The samples (100 to 200 µl) were loaded on a Sepharose 2B (Pharmacia) column (0·7 × 27 cm) in equilibrium with 5 mM-Tris/HCl buffer (pH 7·6) containing 100 mM-NaCl. The flow rate was 4 ml h^{-1}. The DNA, which appeared in the void volume, was collected in a volume of 1 to 2 ml and precipitated with ethanol.

Growth of bacteria. Escherichia coli K12 strain Hb101 (Boyer & Roulland-Dussoix, 1969) was grown in L broth supplemented with glucose (0·1%, w/v). *Escherichia coli* K12 strain χ1776 (Curtiss *et al.*, 1977) was grown in L broth containing glucose (0·1%, w/v), diaminopimelic acid (100 µg ml^{-1}) and thymidine (4 µg ml^{-1}). Amplification of plasmid DNA with chloramphenicol (12·5 µg ml^{-1}) was performed according to Curtiss *et al.* (1977). To determine the viable cell number, bacteria were serially diluted in fresh culture medium without glucose at 37 °C. Portions (100 µl) of three appropriate dilutions were spread evenly over the surface of agar plates (Bacto antibiotic medium 2, Difco) which had been dried at 37 °C for 3 to 4 h. The plates were incubated for 24 to 30 h at 37 °C and colonies were then counted.

Transformation of bacteria. The methods of Cohen *et al.* (1972) and Norgard *et al.* (1978) were used for transformation of strains Hb101 and χ1776, respectively. Clones containing insertions of Py DNA were identified by colony hybridization according to Grunstein & Hogness (1975).

RESULTS AND DISCUSSION

Cloning of HindIII fragments of Py virus DNA into the HindIII site of pBR322

Restriction endonuclease *Hin*dIII cleaves Py virus DNA at 0·018 and 0·46 map units, generating two fragments A and B of molecular weights $2{\cdot}0 \times 10^6$ and $1{\cdot}5 \times 10^6$, respectively (Griffin *et al.*, 1974). The fragments of A2 Py virus DNA were separated by electrophoresis in a 0·7% agarose slab gel, and ligated separately to linear *Hin*dIII-treated pBR322. The products of the ligation reaction were used to transform strain χ1776, and Ap-resistant colonies were screened for a Tc-sensitive phenotype on agar plates. Only a few colonies transformed by the ligation mixture pBR322/*Hin*dIII-B were sensitive to Tc. However, when the colonies were screened for the presence of Py DNA by the method of Grunstein & Hogness (1975), about half of them annealed with the Py DNA probe. Since the plasmid *Hin*dIII site is located within the promoter region for the gene responsible for Tc resistance, insertion of DNA fragments into this site reduces the level of Tc resistance to varying degrees depending on the sequence cloned (Rodriguez *et al.*, 1977; Ullrich *et al.*, 1977).

Some of the colonies which were found to contain Py DNA sequences were propagated in culture and then used to prepare plasmid DNA by the method of Curtiss *et al.* (1977). Four different recombinants were isolated that comprised a full-length Py DNA A or B fragment inserted into the *Hin*dIII site of pBR322 in the two possible orientations. The characterization of these recombinants, as well as their biological properties in permissive and in non-permissive bacteria, have been reported elsewhere (Bastin *et al.*, 1980). The reduction in Tc resistance due to the insertion of Py DNA in the *Hin*dIII site of pBR322 is illustrated in Fig. 1 (*a*). The growth of bacteria carrying recombinant plasmids with an altered *tet* gene was noticeably reduced by Tc in liquid culture. Plasmid pPA8, a recombinant containing the *Hin*dIII-A fragment of Py DNA, conferred the same growth pattern as pPB41, a recombinant containing the *Hin*dIII-B fragment. The orientation of Py sequences within the plasmid appeared to be unimportant because the same growth curves were observed for bacteria carrying recombinants with inserts in opposite orientations to pPA8 and pPB41 (not shown).

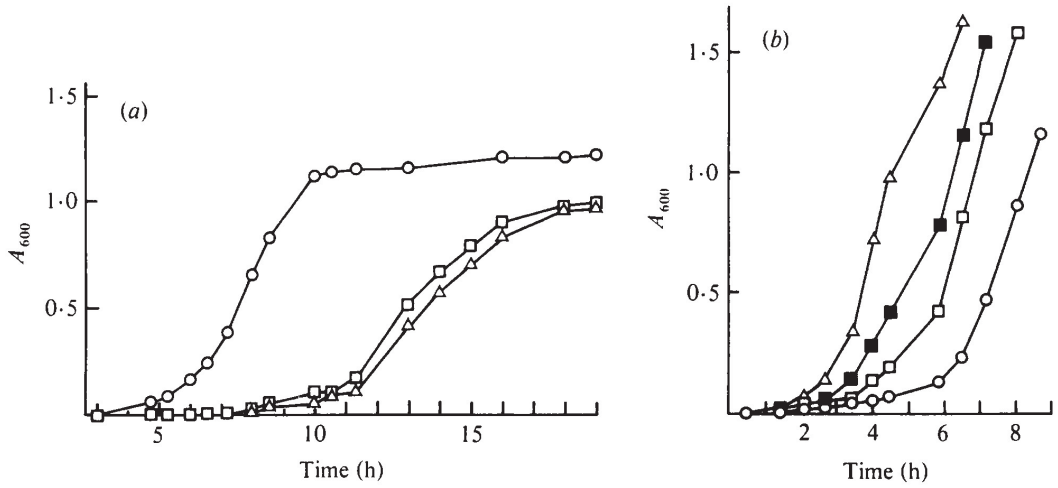

Fig. 1. (*a*) Reduction in Tc resistance of *E. coli* K12 strain χ1776 due to the insertion of Py virus DNA in the *Hin*dIII site of pBR322: pBR322 with unaltered *tet* (○); pBR322 with insertion of fragment *Hin*dIII-A (□); pBR322 with insertion of fragment *Hin*dIII-B (△). Bacteria were grown overnight at 37 °C in the presence of Ap (50 μg ml^{-1}). Cultures were then diluted 100-fold in fresh medium containing Tc (12·5 μg ml^{-1}) and agitated at 37 °C on a rotary shaker (150 rev. min^{-1}). At the time of the dilution (time 0), all stationary phase cultures contained about 71 × 10^6 viable cells ml^{-1}. Bacterial growth was monitored by A_{600} readings.

(*b*) Growth of *E. coli* K12 strain Hb101 harbouring pPE7 monomer (○), pPE7 pentamer (□), pPE7 hexamer (■) and pPE47 monomer (△). Cultures were grown to saturation at 37 °C in the presence of Tc (25 μg ml^{-1}). At time 0, they were diluted 100-fold in fresh medium containing Tc (25 μg ml^{-1}) and agitated at 37 °C on a rotary shaker (150 rev. min^{-1}). Saturation was reached at an A_{600} of 1·8 to 1·9 (not shown).

The appearance of a lag phase preceding any significant increase in turbidity prompted us to use such growth conditions in liquid culture to differentiate recombinant from non-recombinant plasmids. Ap-resistant transformants were grown overnight (16 h) at 37 °C in 1 ml L broth containing 100 μg Ap ml^{-1}. The cultures were then diluted 100-fold in fresh medium supplemented with Tc (12·5 μg ml^{-1}). Cultures of bacteria carrying pBR322 became turbid within 6 to 8 h, but those of strains harbouring recombinant plasmids with altered expression of Tc resistance required over 12 h for the development of visible growth. Under these conditions, all the clones that annealed with the ^{32}P-labelled Py DNA probe in the colony hybridization experiment also showed a prolonged lag in the screening assay. As an additional test, we ligated the *Hin*dIII fragment of P16 DNA (a small plaque variant of Py virus) in pBR322 and transformed χ1776 to Ap resistance in a 'shot gun' experiment. Fifty colonies were then screened for the altered Tc-resistant phenotype. Eight of them, displaying the phenotype, were propagated in culture and were found to contain either the *Hin*dIII-A or the *Hin*dIII-B fragment of the P16 strain of Py virus. Although our procedure involving the partial inactivation of the *tet* gene is not as powerful as the colony hybridization procedure of Grunstein & Hogness (1975), it is very simple and rapid, and yet sufficiently reliable for differentiating recombinant from non-recombinant plasmids. It is especially useful when the DNA to be inserted consists of only few molecular species, such as fragments of pure virus DNA, that do not have to be identified in a heterologous population of molecules by use of a specific probe.

Use of the altered tet gene for selection

Our interest in the altered *tet* gene as a selection marker stemmed from the need to produce plasmids, with partially inactivated *tet*, that no longer conferred Ap resistance. Such plasmids

were constructed from recombinants pPA8 and pPB41 (Bastin *et al.*, 1980) by circularization of their largest *Pst*I fragments in order to obtain clones containing only parts of the early region of the Py genome. Plasmid pPE7 (mol. wt $3{\cdot}136 \times 10^6$), the recombinant derived from pPA8, contained the Py sequence comprised between 0·797 and 0·018 map units. Plasmid pPE47 (mol. wt $2{\cdot}825 \times 10^6$), the recombinant derived from pPB41, contained the sequences comprised between 0·018 and 0·150 map units. The details concerning the construction and characterization of these plasmids will be described elsewhere. As both pPE7 and pPE47 were deprived of their *amp* gene, the possibility of using the altered *tet* gene for selection was investigated by transforming strain χ1776 to Tc resistance. Very small colonies appeared on the agar after 3 d incubation at 37 °C. All colonies were Ap sensitive, an indication that part of the *amp* gene had been deleted. Clones, especially those transformed by pPE7, were not easily propagated in liquid culture even at Tc concentrations of 5 to 6 μg ml^{-1}. However, plasmid DNA could be isolated from some transformants carrying pPE7 or pPE47 and was used to transform the *recA*$^-$ *E. coli* K12 strain Hb101. Larger amounts of plasmid DNA could be isolated from strain Hb101 than from χ1776 and it was thought that this strain, harbouring plasmids pPE7 or pPE47, would grow better than χ1776 in the presence of Tc. The DNA from several independent transformants was isolated according to the method of Birnboim & Doly (1979). Agarose gel electrophoresis showed that less than half of the clones contained the expected supercoiled and open circular forms of the DNA. The other clones contained slower migrating DNAs that were identified as multimers of plasmids pPE7 and pPE47 generated in χ1776, a *rec*$^+$ host, and cloned as single species in the *recA*$^-$ host. The details concerning the cloning and characterization of such multimers will be described elsewhere.

We expected that the formation of plasmid multimers might increase the resistance level to Tc as a result of a gene dosage effect. To investigate this possibility, we determined the minimal inhibitory concentration (m.i.c.) of Tc for strain Hb101 harbouring the various multimers of both pPE7 and pPE47. An m.i.c. value of about 45 μg ml^{-1} was found for all strains except those transformed by pPE7 monomer for which the m.i.c. was 20% lower. Fig. 1 (*b*) shows the growth in Tc broth of strains carrying pPE7 monomer, pentamer or hexamer or pPE47 monomer. All bacteria grew with the same generation time of about 55 min. However, the lag phase was substantially reduced for bacteria harbouring pPE47 or plasmid multimers of pPE7. The higher Tc resistance of clones carrying pPE47 compared with that of strains harbouring pPE7 is an indication that the structure of the DNA in the promoter region may be important. However, the expression of the Tc resistance gene cannot be related only to the structure of the DNA inserted (Rodriguez *et al.*, 1977; Ullrich *et al.*, 1977) or to the polarity of insertion as has been shown by Mooi *et al.* (1979). A higher level of resistance can be achieved in some bacteria as a result of a gene dosage effect due to the expression of multiple copies of a low level Tc resistance gene. Covarrubias *et al.* (unpublished) found such a gene dosage effect when the Tc resistance gene of pBR313 was integrated into a plasmid which was present in only a few copies per cell. We found that resistance to Tc was indeed affected by the formation of plasmid multimers, but essentially at the level of the induction period, and thus conceivably at a very critical time for the development of a bacterial cell with an altered *tet* into a colony.

The formation of multimers did not result in any significant change in net plasmid production. After amplification with chloramphenicol, we routinely obtained about 1·5 mg supercoiled DNA per litre culture for both monomeric and multimeric forms of pPE7 and pPE47.

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