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The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation

(Recombinant DNA; filamentous bacteriophage M13; polylinkers; β-galactosidase; α-complementation selection; chimeric genomes)

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SUMMARY

The versatility of insertional inactivation of β-galactosidase activity for subcloning and sequencing has been enhanced by combining a chemically synthesized oligonucleotide which specifies nine 6-bp-cutter restriction sites including *Bgl*II, *Xho*I, *Nru*I, *Cla*I, *Sac*I and *Eco*RV in various configurations with existing polylinkers to create a set of highly versatile cloning sites. These improved polylinkers have been inserted into plasmids (the pICs) for routine cloning of double-stranded DNA, and into chimeric phage/plasmids (the pICEMs) for biological production of single stranded DNA. The most versatile polylinker specifies 17 restriction sites in the β-galactosidase α-complementing gene fragment. One of the new polylinkers was inserted into M13 DNA to produce a vector (M13mIC7) with nine cloning sites.

INTRODUCTION

The insertional inactivation of β-galactosidase α-complementation is a powerful method for identifying recombinant DNA molecules in plasmids and phage. The ability of the gene fragment to tolerate extensive modification has permitted the engineering of polylinkers in the alpha donor region of the pUC plasmids (Vieira and Messing, 1982) and M13 bacteriophage (Messing and Vieira, 1982). We have extended the versatility of these vectors by combining an oligonucleotide which specifies nine 6-bp-cutter restriction enzyme sites in various configurations with existing polylinkers to construct a set of greatly improved cloning sites. These have been introduced into plasmids and into chimeric phage/plasmids for biological production of single stranded DNA.

MATERIALS AND METHODS

(a) Bacterial strains

The pUC, pIC and pICEM plasmids were grown in the *Escherichia coli* K-12 strain JM83 (*ara*, *Δlac*-

Abbreviations: Ap, ampicillin; *bla*, β-lactamase; bp, base pairs; dsDNA/ssDNA, double- and single-stranded DNA respectively; *ori*, origin of replication; IC, Irvine California; $^{r\ R}$ (superscript), resistance.

pro, strA, thi, ϕ80d*lacZ*ΔM15) (Vieira and Messing, 1982). The pUC plasmids were kindly provided by J. Messing and the pEMBL vectors were kindly provided by Dr. F. Hagen of Zymos Corp. The M13mp10 is described by Messing (1983). GM33 *dam*-3, F^-, λ^- (Marinus and Morris, 1973) was a kind gift of Dr. K. Bertrand, University of California, Irvine (CGSC No. 5126).

(b) Procedures

DNA was prepared by the cleared lysate method (Katz et al., 1973), phenol-extracted and loaded on a 250-ml Biogel A-50M, 100–200 mesh column equilibrated with 500 mM NaCl, 20 mM Tris · HCl pH8 and 1 mM EDTA. Excluded fractions containing plasmid and chromosomal DNA were banded in a CsCl density equilibrium gradient for final purification. Restriction enzyme digests employed a core buffer (6 mM Tris · HCl pH 7.4, 6 mM $MgCl_2$ and 6 mM mercaptoethanol) with appropriate salt. DNA fragments were purified from gels by soaking the excised bands overnight in GEB buffer (10 mM Tris · HCl pH 8, 500 mM NaCl, 1 mM EDTA) followed by removal of polyacrylamide and ethanol precipitation. Standard procedures were performed as described by Maniatis et al. (1982).

RESULTS AND DISCUSSION

(a) Construction of pIC7 and mIC7

Two complementary 33-mer oligonucleotides were synthesized by the hindered dialkylamino phosphite method on functionalized "long chain alkyl amine" controlled pore glass supports (Pierce Chemical Co.) utilizing diisopropyl phosphoramidites. A 20-min synthesis cycle was used similar to that described by Adams et al. (1983). After deprotection and cleavage from the support, the 33-mers were purified by polyacrylamide gel electrophoresis. After elution from the gel, the fragments were ethanol-precipitated at −70 °C and redissolved in deionized water for subsequent annealing and cloning. The sequences of the two 33-mers are 5′-AATTCATCGATATCTAGATCTCGAGCTCGCGAA-3′ and 5′-AGCTTTCGCGAGCTCGAGATCTAGATATCGATG-3′. After annealing, the double-stranded oligonucleotide which has *Eco*RI and *Hin*dIII cohesive ends was ligated to *Eco*RI and *Hin*dIII cut pUC8 and M13mp10. Blue colonies or plaques were selected and designated pIC7 and M13mIC7. The structure of the new cloning site in pIC7 was confirmed by restriction analysis and that of M13mIC7 by nucleotide sequence analysis (not shown).

(b) Construction of pIC19R, pIC19H, pIC20R, and pIC20H

The new polylinker of pIC7 was linked to the polylinkers both pUC9 and pUC19 in two orientations thus providing approx. 14 unique restriction enzyme sites in the *lacZ* gene region. The polylinkers were fused by cutting pIC7 with *Hin*dIII and *Nar*I eluting the large fragment bearing the *ori*, Ap^R and polylinker from an acrylamide gel and ligating to the small *Nar*I-*Hin*dIII fragment from pUC9 to give pIC19R or from pUC19 to give pIC20R. The Ap^R, Lac^+ colonies were selected and the structures of pIC19R and pIC20R confirmed by restriction analysis (Fig. 1).

The reciprocal constructions involved ligating the small *Eco*RI-*Nar*I fragment of pIC7 to the large *Eco*RI-*Nar*I fragment of pUC9 and pUC19, respectively. The structures of the resulting pIC19H and pIC20H vectors are shown in Fig. 1.

(c) Mapping of constructs

The structure of the polylinker in the new vectors was confirmed by cutting each vector with *Bgl*I which cuts twice (one cut is 150 bp from the linker site) and again with each of the enzymes specified by the linker region. The digests were analyzed by gel electrophoresis. The observed fragment sizes are those expected from the sequence. For example the *Cla*I, *Eco*RV, *Bgl*II, *Xho*I, *Sac*I and *Nru*I sites all map approx. 5 bp apart while the *Sma*I, *Bam*HI, *Sal*I and *Pst*I sites map 7–9 bp apart as indicated by the sequence. Similar mapping performed on each of the constructs confirmed the localization and order of the restriction sites in the linker regions.

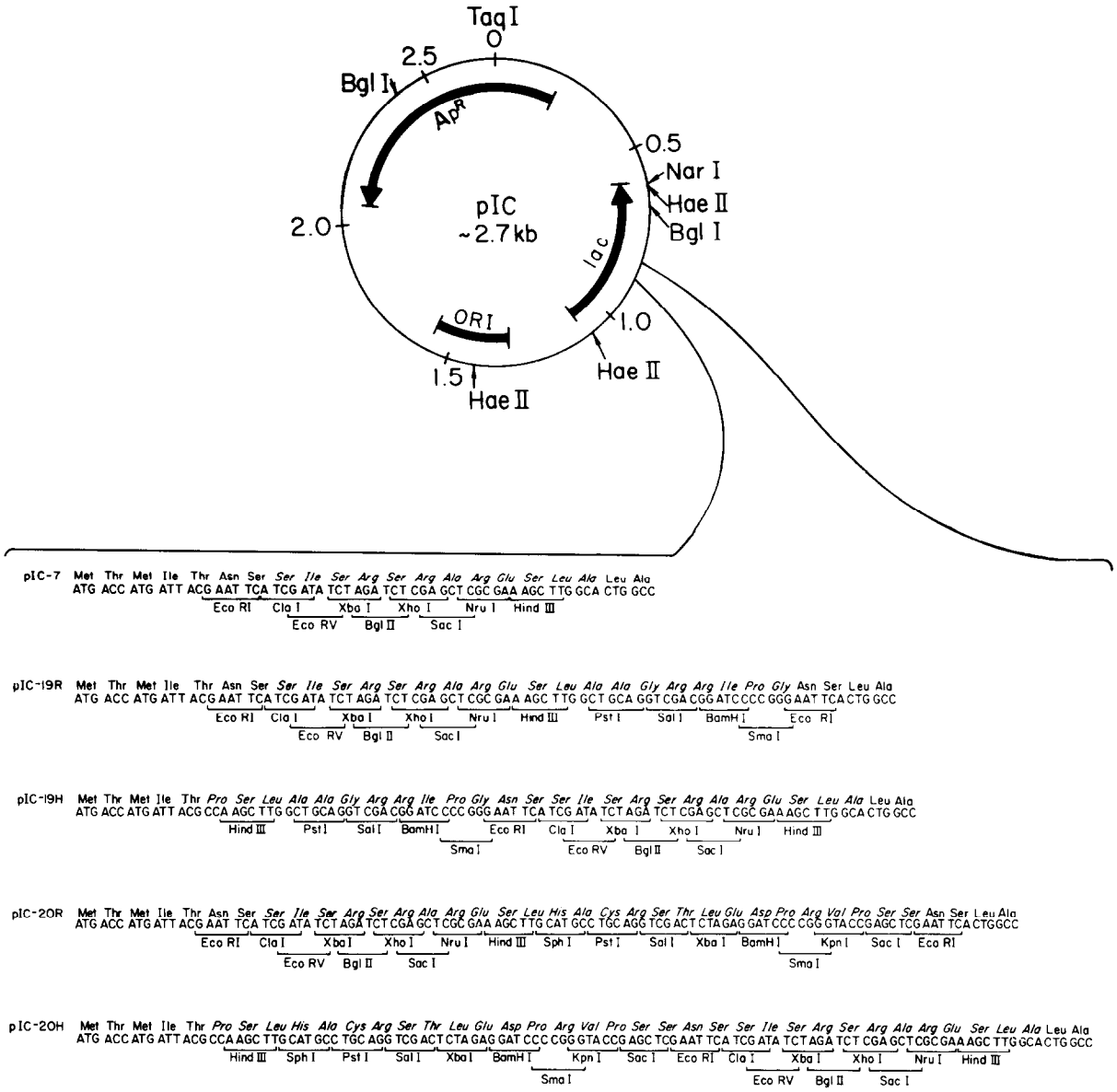

Fig. 1. Structure of pIC plasmid vectors. The *Taq*I site (at 4018 in pBR322) is used as the zero reference point. Other *Taq*I sites are located between the *Hae*II sites (2352 and 2722 on pBR322 map) and in the polylinker. The sequence of the polylinker is shown starting with the ATG codon of the β-galactosidase gene and ending with the natural *Hae*III site and the alanine of codon 8 to the right. The conceptual translation of the sequence is presented with the additional amino acids contributed by the inserted polylinker sequences shown in italics. The cloning site of M13mIC7 is the same as that shown for pIC7.

(d) Construction of pICEM19R+; pICEM19R−; pICEM19H+; pICEM19H−

Chimeric phage/plasmids bearing the origin of replication from a single stranded phage in a plasmid combine the advantages of single stranded phage small *Bgl*I fragments of pEMBL8+ and 8− bearing the f1 replicons in either orientation (Dente et al., plasmids (e.g., Dotto et al., 1981; Dotto and Horiuchi, 1981). Thus we combined the improved cloning sites of the pIC vectors with the f1 origin of the pEMBL vectors (Dente et al., 1983).

We ligated the large fragment of *Bgl*I-cut pIC7 bearing the *lacZ* gene and part of the Ap^R gene to the small *Bgl*I fragments of pEMBL 8+ and 8− bearing the f1 replicons in either orientation (Dente et al.,

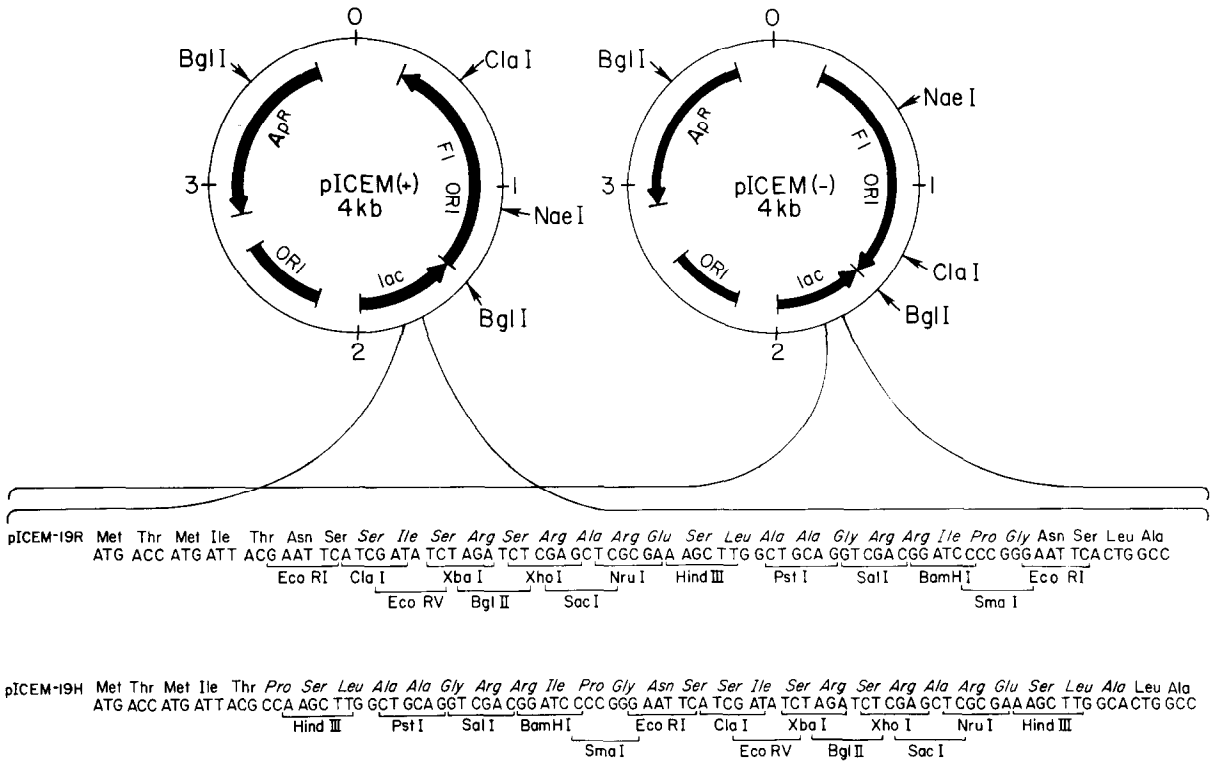

Fig. 2. Structure of pICEM chimeric phage vectors. The reference point is the same as that used by Dente et al. (1983) for the pEMBL vectors and corresponds to the original *Eco*RI site of pBR322 which has been modified during construction of pUC8. The sequence of the polylinker is shown starting with the ATG codon of the *lacZ* gene and ending with the natural *Hae*III site and the alanine of codon 8 to the right. The conceptual translation of the sequence is presented with the additional amino acids contributed by the inserted polylinker sequences shown in italics.

1983) and the remainder of the Ap^R gene and selected Ap^R, Lac^+ colonies. Plasmids bearing the polylinker of pIC19R and pIC19H linked to the f1 replicon in both the plus and minus strand orientation were constructed (designated pICEM19R+; pICEM 19R−; pICEM19H+ and pICEM19H−) (Fig. 2). The structures were confirmed by restriction analysis as described above. Confirmation of pICEM19+ and 19R− is shown in Fig. 3. The presence of a *Cla*I site in the f1 fragment of the pEMBL vectors compromises the use of this enzyme in these vectors. However, use of smaller *ori* fragments could avoid unwanted restriction sites in the future.

(e) Modification of the *Xba*I site

The terminal A of the TCTAGA *Xba*I site in pIC7 falls within the *dam* methylase recognition sequence GATC. When grown in a methylase deficient host (GM33, *dam*-3), the *Xba*I site shows normal cutting in standard restriction reactions but when grown in JM83, cutting is blocked completely (not shown). Thus hemimethylation of the terminal A blocks cleavage of *Xba*I (Gruenbaum et al., 1981). The sensitivity of this plasmid to *Xba*I digestion provides a sensitive test for the state of the *dam* methylase system in a strain. Additionally, one can mask or unmask this site during complex constructions by the appropriate choice of host bacterium.

(f) Other vectors

The polylinkers described here can now be readily mobilized with either *Eco*RI or *Hin*dIII or a double digest and if desired they can be inserted into the symmetrical *Eco*RI or *Hin*dIII sites of the pIC and pICEM vectors to generate other polylinker configurations. We anticipate improvements in the design of the chimeric phage/plasmids to possibly produce

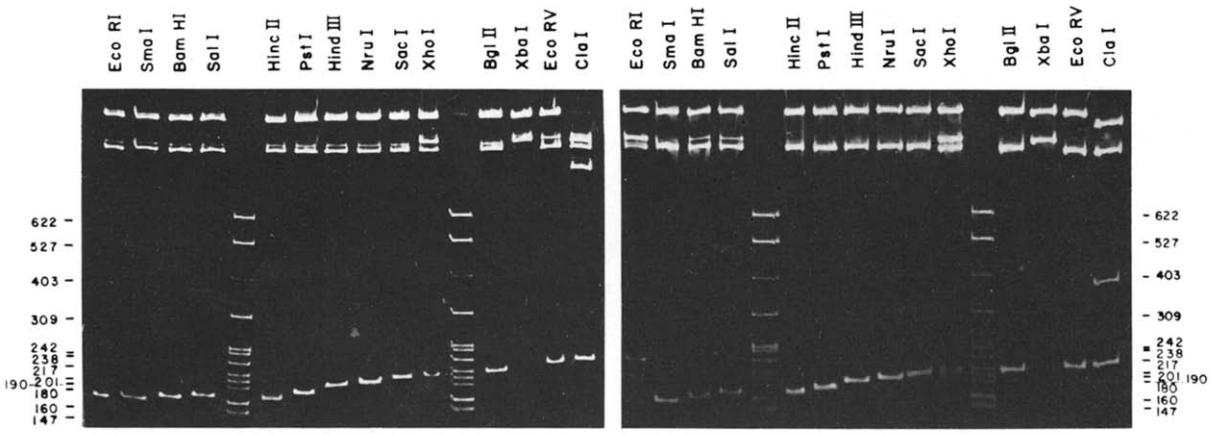

Fig. 3. High-resolution electropherograms of pICEM19R+ and pICEM19R–. Plasmid pICEM19R+ is shown in the left panel and pICEM19R– is shown on the right. Each vector was cut with *Bgl*I and with a second enzyme in the polylinker using a core buffer and added salt as indicated in MATERIALS AND METHODS, section **b**. Electrophoresis was for 2 h at 150 V on a 4% polyacrylamide gel in 89 mM Tris·borate, 89 mM borate; 2 mM EDTA pH 8. The small fragments are a measure of the distance from the *Bgl*I site to the site of the polylinker. All fragments run between 150 and 220 bp as expected. Size standards are *Hpa*II-cut pBR322. *Xba*I does not cut due to *dam*$^+$ methylation.

higher yields of chimeric phage and permit more flexibility in vector design. The *Nar*I site of the pIC plasmids provides a convenient site for future insertion of smaller phage *ori* fragments.

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