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An improved positive selection plasmid vector constructed by oligonucleotide mediated mutagenesis

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ABSTRACT

An Escherichia coli plasmid vector, pUN121, has been constructed which allows for positive selection of transformants harboring DNA inserts. The vector is based on plasmid pTR262 (Roberts et al, Gene, 12, (1980), 123-127) in which the tetracycline resistance gene is under transcriptional control of the repressor protein coded by the phage lambda cI gene. This plasmid has been rearranged, using in vitro recombinant techniques including oligonucleotide mediated mutagenesis to yield a smaller plasmid (4.4 kb) with unique cloning sites for EcoRI, XmaI and SmaI in addition to the unique HindIII and BclI sites. The plasmid has a functional ampicillin resistance gene and the new restriction sites (EcoRI, XmaI and SmaI) when used for cloning, give rise to tetracycline resistant transformants.

INTRODUCTION

Recombinant DNA operations are simplified using plasmid vectors which allow positive selection for transformants containing DNA inserts. Several such vectors have been constructed for Escherichia coli (1,2,3,4,5,6) and recently for Bacillus subtilis (7). Unfortunately, all these vectors have a low number of sites available for cloning which limits their usefulness. In addition, some need special host strains (3) and others are too large for cloning (2,5).

Here we describe a method to expand the number of available cloning sites. Oligonucleotide-mediated in vitro mutagenesis is used to create specific base transversions in the coding sequence of a structural gene, thus creating new restriction sites without altering the amino acid sequence of the encoded polypeptide. We have used this technique to construct the cloning vehicle pUN121 in which the phage lambda cI (8) gene has been mutated creating new unique cloning sites for EcoRI and

XmaI/SmaI. The tetracycline resistance gene derived from pTR262 is transcribed from the right promoter of phage lambda (1,9) and therefore under control of the repressor protein coded by the cI gene. Normally, E.coli cells harboring pUN121 are sensitive to tetracycline since the cI protein represses transcription from the gene. However, insertion of DNA fragments into any one of the five cloning sites (HindIII, BclI, EcoRI, SmaI and XmaI) inactivates the repressor gene yielding tetracycline resistant transformants.

MATERIALS AND METHODS

Bacterial strains, phage and plasmids

E.coli HB101 (10) and LE392 (11) were used as bacterial hosts. Phage lambda W30 (a cI mutant kindly supplied by Leif Isaksson) was used. The plasmid vectors were pTR262 (1), pBR322 (12), pBR328 (13) and R1 (14).

DNA constructions

Restriction enzymes (New England Biolabs and Boehringer, Mannheim), T4-DNA Ligase (New England Biolabs) and DNA Polymerase I Large Fragment (New England Biolabs) were used according to the suppliers recommendations. Transformation of competent E.coli was made according to Morrison (15). Antibiotic resistant clones were selected on rich medium plates containing ampicillin (100 μg/ml), tetracycline (8 μg/ml) and/or kanamycin (50 μg/ml). Plasmid DNA was purified according to the method described by Birnboim and Doly (16). The PstI linker (5'-GCTGCAGC-3') was supplied by New England Biolabs.

Synthesis of oligodeoxyribonucleotides

The oligomers were synthesized automatically by a machine developed by KabiGen AB following published procedures (17,18) and 2-dimensional homochromatography was carried out according to Tu and Wu (19).

Preparation of single stranded DNA

Plasmid DNA was nicked by incubating 20μg in 100 μl buffer (10 mM Tris.HCl pH 7.4 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) containing 60μg/ml ethidium bromide and 5U BamHI (Boehringer, Mannheim) for 3 hours at 18^0C. Approximately 70% of the ccc-DNA was nicked. 10 μg of nicked plasmid was treated with 280U

Exonuclease III (New England Biolabs) for 45 minutes at 37°C according to the recommendations of the supplier. The reaction mixture was extracted with phenol and the water phase was chromatographed on Sephadex G-50 in a syringe (20) in a high salt TES buffer (50 mM Tris.HCl pH 7.4, 1 mM EDTA, 1 M NaCl).

Oligonucleotide mediated mutagenesis

The nicked and exonuclease treated plasmid DNA was mixed with 20 pmol oligonucleotide in a buffer containing 0,5 M NaCl in a volume of 5µl. The mixture was heated to 70°C for 5 minutes cooled to room temperature and then transferred to an ice-water bath. Buffer and salt concentrations were adjusted to 100 mM NaCl, 20 mM Tris.HCl pH7.4, 10 mM DTT, 10 mM $MgCl_2$, 0,5 mM dNTPs and 0,5 mM ATP. DNA polymerase I Large Fragment (50U) and T4 DNA Ligase (40U) were added to a final volume of 50µl. The reaction mixture was incubated 11 hours at 18°C and transformed into competent E.coli HB101.

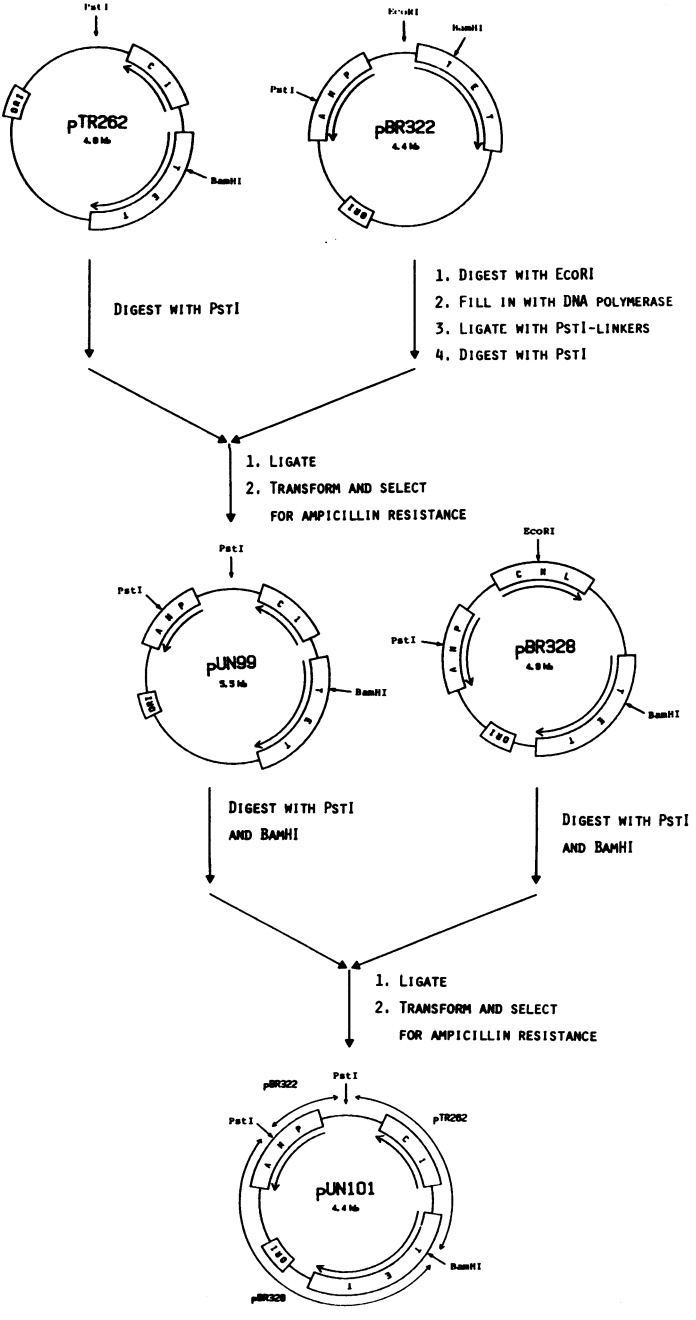
RESULTS

Reconstruction of the ampicillin resistance gene

The plasmid pTR262 (1) is convenient for cloning operations including constructions of DNA libraries (unpublished results) but it lacks an intact antibiotic resistance gene. Transformants with this plasmid can only be selected by resistance to an antibiotic when an insert is present. We decided first to restore the ampicillin resistance gene which is partly deleted in this plasmid. The cloning vector pUN99 was constructed as shown in Fig. 1. Plasmid pBR322 (12) was digested with EcoRI and the protruding ends filled in with DNA polymerase I (large fragment). PstI-linkers were ligated to the blunt-ends followed by digestion with PstI. This gave two fragments, one consisting of the 5'-end of the ampicillin resistance gene. The fragments were ligated to pTR262 which had been digested with PstI and transformed into E.coli HB101. Ampicillin resistant tetracycline sensitive clones were isolated. The resulting plasmid, pUN99, contains a functional ampicillin resistance gene (Fig. 1). The size of pUN99 is approximately 5.5 kb.

Construction of pUN101

Attempts were then made to reduce the size of the vector.


PstI
pTR262
4.8 kb
BamHI
EcoRI
BamHI
PstI
pBR322
4.4 kb
DIGEST WITH PstI
1. DIGEST WITH EcoRI
2. FILL IN WITH DNA POLYMERASE
3. LIGATE WITH PstI-LINKERS
4. DIGEST WITH PstI
1. LIGATE
2. TRANSFORM AND SELECT
FOR AMPICILLIN RESISTANCE
PstI
PstI
pUN99
5.3 kb
BamHI
EcoRI
PstI
pBR328
4.9 kb
BamHI
DIGEST WITH PstI
AND BamHI
DIGEST WITH PstI
AND BamHI
1. LIGATE
2. TRANSFORM AND SELECT
FOR AMPICILLIN RESISTANCE
pBR322
PstI
PstI
pTR262
pUN101
4.4 kb
BamHI
pBR328

Soberon et al. (13) showed that a 1089bp fragment of pBR322 can be deleted without destroying the resistance genes or the replication function. Instead of deleting this region from pUN99, we used the plasmid, pBR328 which already lacks this region in the construction of the vector pUN101. As outlined in Fig. 1 both pUN99 and pBR328 were digested with PstI and BamHI. After ligation and transformation the clones were screened for ampicillin resistance as well as chloramphenicol and tetracycline sensitivity. Such clones were, of course, isolated in a background of the original plasmid pUN99. Plasmid pUN101 is schematically shown in Fig. 1 and its size is around 4.4 kb.

Synthesis of oligonucleotides

Oligonucleotide mediated mutagenesis was used to create new restriction sites for cloning in pUN101. As EcoRI (GAATTC) is used frequently in cloning experiments and SmaI (CCCGGG) would allow cloning of blunt-end fragments, these two sites were introduced. Since SmaI sites are recognized also by XmaI an additional site would be gained.

The 711 nucleotide sequence of the cI structural gene (8) was computer scanned for possible mutations creating the restriction sites mentioned above without altering the amino acid sequence. In Fig. 2 the three possible sites for EcoRI and two for SmaI are shown. We decided to create the EcoRI site at nucleotide 226 (Fig. 2) because only one mismatch is involved. The tetradecamer 5'-GAAGGGCTGAATTC-3' was synthesized giving a G/T mismatch (Fig. 3). Among the two possible SmaI sites the sequence at nucleotide 529 (Fig. 2) was chosen because it is flanked by the HindIII and BclI sites approximately 50 nucleotides away on each side. Therefore, any blunt-end fragment ligated into the SmaI site can be excised using these two enzymes. The synthesized tetradecamer, 5'-GAAATCCCCGGGCT-3', has two mismatches with the corresponding plasmid DNA sequence, an

Fig. 1 Construction of positive selection plasmid pUN101
Relevant restriction sites are indicated. Boxes show the relative positions of genes coding for the lambda phage repressor (cI), β-lactamase (AMP), tetracycline resistance (TET) and chloramphenicol resistance (CML). The arrows indicate the orientation of the genes and the replication origin is marked ORI.

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ATGAGCACAAAAAAGAAACCATTAACACAAGAGCAGCTTGAGGACGCACGTCGCCTTAAA      60
MetSerThrLysLysLysProLeuThrGlnGluGlnLeuGluAspAlaArgArgLeuLys

GCAATTTATGAAAAAAAGAAAAATGAACTTGGCTTATCCCAGGAATCTGTCGCAGACAAG     120
AlaIleTyrGluLysLysLysAsnGluLeuGlyLeuSerGlnGluSerValAlaAspLys

ATGGGGATGGGGCAGTCAGGCGTTGGTGCTTTATTTAATGGCATCAATGCATTAAATGCT     180
MetGlyMetGlyGlnSerGlyValGlyAlaLeuPheAsnGlyIleAsnAlaLeuAsnAla

                                                  GAATTC
TATAACGCCGCATTGCTTGCAAAAATTCTCAAAGTTAGCGTTGAAGAATTTAGCCCTTCA     240
TyrAsnAlaAlaLeuLeuAlaLysIleLeuLysValSerValGluGluPheSerProSer

    CCCGGG
ATCGCCAGAGAAATCTACGAGATGTATGAAGCGGTTAGTATGCAGCCGTCACTTAGAAGT     300
IleAlaArgGluIleTyrGluMetTyrGluAlaValSerMetGlnProSerLeuArgSer

GAGTATGAGTACCCTGTTTTTTTCTCATGTTCAGGCAGGGATGTTCTCACCTGAGCTTAGA     360
GluTyrGluTyrProValPheSerHisValGlnAlaGlyMetPheSerProGluLeuArg

ACCTTTACCAAAGGTGATGCGGAGAGATGGGTAAGCACAACCAAAAAAGCCAGTGATTCT     420
ThrPheThrLysGlyAspAlaGluArgTrpValSerThrThrLysLysAlaSerAspSer

                    GAATTC                                  AAGC
GCATTCTGGCTTGAGGTTGAAGGTAATTCCATGACCGCACCAACAGGCTCCAAGCCAAGC     480
AlaPheTrpLeuGluValGluGlyAsnSerMetThrAlaProThrGlySerLysProSer

TT                                                  CCCGGG
TTTCCTGACGGAATGTTAATTCTCGTTGACCCTGAGCAGGCTGTTGAGCCAGGTGATTTC     540
PheProAspGlyMetLeuIleLeuValAspProGluGlnAlaValGluProGlyAspPhe

                    GAATTC              TGATCA
TGCATAGCCAGACTTGGGGGTGATGAGTTTACCTTCAAGAAACTGATCAGGGATAGCGGT     600
CysIleAlaArgLeuGlyGlyAspGluPheThrPheLysLysLeuIleArgAspSerGly

CAGGTGTTTTTACAACCACTAAACCCACAGTACCCAATGATCCCATGCAATGAGAGTTGT     660
GlnValPheLeuGlnProLeuAsnProGlnTyrProMetIleProCysAsnGluSerCys

TCCGTTGTGGGGAAAGTTATCGCTAGTCAGTGGCCTGAAGAGACGTTTGGC              711
SerValValGlyLysValIleAlaSerGlnTrpProGluGluThrPheGly
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Fig. 2 Nucleotide and amino acid sequence of the cI gene
Mutation alternatives to create an EcoRI restriction site (GAATTC) or a SmaI site (CCCGGG) are indicated as well as the existing HindIII (CTGCAG) and BclI (TGATCA) sites. Underlined sequences represent sequence after mutation.

A/G and a T/C (Fig. 3).

Oligonucleotide mediated mutagenesis

To achieve specific mutagenesis oligonucleotides were annealed to plasmid DNA before they were extended with DNA polymerase. The resultant plasmids were screened for EcoRI sites. Plasmid pUN101 was converted to single stranded circles

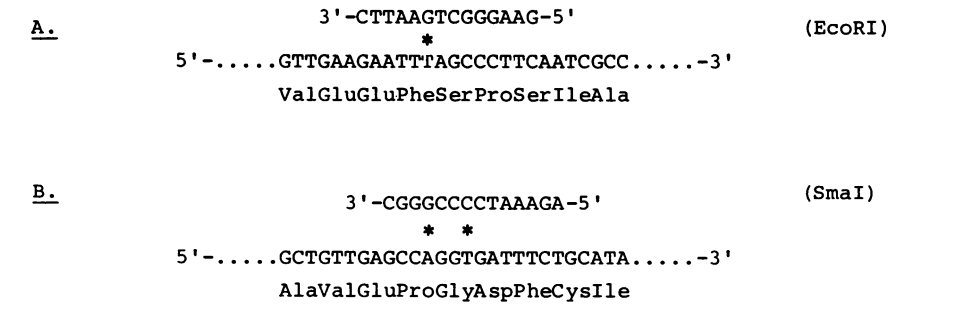

Fig. 3 Nucleotide sequence of synthesized oligonucleotide and the corresponding viral sequence at the point of mutagenesis. A star indicates a non-complementary base pair.
A. Dodecamer primer used to create the EcoRI recognition site.
B. Dodecamer primer used to create the SmaI/XmaI recognition site.

by site specific nicking using BamHI in an ethidium bromide buffer (21) followed by exonuclease III treatment. The 5'-phosphorylated EcoRI-tetradecamer was annealed to the single-stranded plasmid and elongated using DNA polymerase I (large fragment). After transformation to E.coli, approximately 30.000 ampicillin resistant clones were pooled and the plasmids were isolated. The total plasmid mixture was digested with EcoRI and ligated together with a 6kb EcoRI-fragment, coding for kanamycin resistance originating from the low copy number plasmid R1 (14). After a second transformation, tetracycline and kanamycin resistant clones were isolated. Restriction analysis showed a 6kb EcoRI insert into the cI gene of pUN101. After digestion with EcoRI and religation, a plasmid designated pUN111 was isolated lacking the 6kb insert. To confirm that the clone harboring pUN111 produces a functional lambda repressor, cross streaking against a strain of phage lambda with a defective cI repressor was performed. The clone was resistant to the phage in contrast to a control clone harboring pBR322.

A second in vitro mutagenesis was performed as above using single stranded pUN111 as template and the SmaI-oligonucleotide (Fig. 3) as primer. After transformation of the DNA approximately 30.000 clones were pooled. The purified plasmid

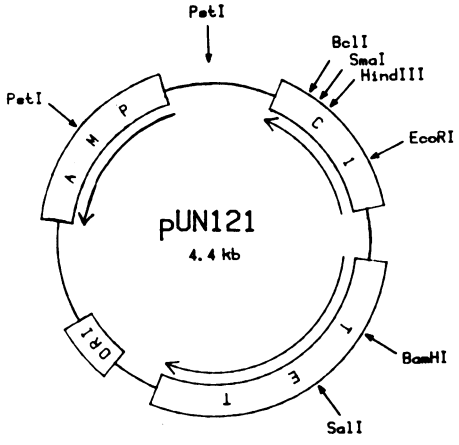

Fig. 4 Schematic drawing of the positive selection vector pUN121
The abbreviations are as in Fig. 1.

mixture was cleaved with XmaI and thereafter mixed with adenovirus DNA digested with the same enzyme. XmaI recognizes the same sequence as SmaI, but creates 5' protruding ends instead of blunt ends. After ligation and transformation tetracycline resistant clones were isolated and restriction analysis showed different XmaI inserts into the cI gene of pUN111. Digestion with XmaI, religation and transformation yielded a clone without a XmaI insert. This plasmid, designated pUN121 is shown in Fig. 4. It has five unique cloning sites in the repressor gene (HindIII, BclI, EcoRI, SmaI and XmaI). Cross streaking against phage lambda (λ cI) confirmed that the modified cI gene codes for a functional lambda repressor.

The new cloning sites were tested by ligating digested pUN121 with EcoRI, XmaI, or SmaI respectively, to adenovirus 2 DNA digested with EcoRI, XmaI or HaeIII.
The new sites gave transformation frequences approximately 10-50 times higher than background (Table 1).

DISCUSSION

Roberts et al. (1) described a cloning vector based on the repressor protein coded by the phage lambda cI gene. The tetracycline resistance gene of this plasmid, pTR262, is under trans-

TABLE 1

Selection for inserted DNA in the new cloning sites of pUN121

Site tested	Adenovirus 2 DNA (μg/ml)	Frequency of tet-r transformants (transformants/ μg pUN121)
EcoRI	0	0.22×10^4
	2	2.9×10^4
	20	9.2×10^4
XmaI	0	0.17×10^4
	2	2.7×10^4
	20	6.8×10^4
SmaI	0	0.11×10^4
	2	0.68×10^4
	20	1.24×10^4

Each reaction mixture contained linearised pUN121 with EcoRI, XmaI or SmaI respectively at a concentration of 20μg/ml. Donor DNA was adenovirus 2 DNA digested with EcoRI, XmaI or HaeIII (blunt end). The ligation mixtures were incubated for 3 hours at 20°C for EcoRI/XmaI and 15 hours at 12°C for SmaI/HaeIII. The ligation mixtures were then transformed into competent E.coli HB101.

criptional control of the repressor molecule. Transformants harboring the plasmid vector have a tetracycline sensitive phenotype but the clones harboring plasmid, with inserted DNA into the repressor gene are resistant. A 100-fold increase in the number of tetracycline resistant transformants was obtained when HindIII- or BclI- generated fragments were ligated into pTR262 digested with these enzymes (1) which agree with our experience (unpublished results). Although, this vector is useful it has disadvantages. First, the plasmid vector itself does not confer antibiotic resistance. Second, the vector contains only two restriction sites, HindIII and BclI in the repressor gene, none generating blunt-end fragments and thirdly the BclI site cannot be used in most E.coli strains due to dam methylation. All these facts limit the use of the vector.

By using oligonucleotide mediated mutagenesis and other in vitro recombinant techniques we have constructed plasmid pUN121 based on this plasmid. The new plasmid is smaller, contains a functional ampicillin resistance gene and has three additional

cloning sites (EcoRI, SmaI and XmaI), the latter, of course, arising due to overlapping sequences with SmaI. The four restriction sites with protruding ends (HindIII, BclI, XmaI and EcoRI) give transformation frequencies approximately 40-100 times higher than ligating only vector molecules (Table 1). The background frequency is only 1-3 per cent when screening our plasmid libraries (data not shown). In contrast, ligation of blunt-end fragments into the SmaI site of pUN121 gives lower transformation frequencies reflecting the low efficiency of the ligation. The best conditions gave, however, around 5-10 times more transformants than background. This is considerable higher than using the pBR322 vector and our vector may therefore be preferred for cloning of blunt-end fragments. As the SmaI site is flanked on both sides by the unique restriction enzymes HindIII and BclI the insert may be recovered flanked by approximately 50 nucleotides of vector DNA on each side.

The technique to expand the number of restriction sites in a gene without altering the deduced amino acid sequence from the nucleotide sequence has obviously not been used previously. A complementary oligonucleotide to one of the alternatives was synthesized with the required mutation introduced, giving one or a few mismatches. Although tetradecamers with one or two mismatches were successfully used here the efficiency of mutagenesis can probably be increased. First, longer oligonucleotides may be used making site specific hybridization more efficient; second, single-stranded phage vectors like M13 (22) or ØX174 (23) can be used as templates instead of double-stranded plasmids converted to single-stranded circles. A third improvement would be to perform the mutagenesis in several successive mutagenesis steps using the DNA of the first step as template for the next (24). When introducing restriction sites into a plasmid it may be advantageous to cleave the resulting plasmid with the enzyme and isolate the appropriate fragment from an agarose gel. As the DNA polymerases have difficulties to pass over origins of replication the yields may also be increased by using so-called gap-duplex DNA as template (25) containing double-stranded template with a single-stranded gap in the region for mutagenesis. Finally in order to randomize

the DNA repair in the mismatch region the template should be prepared in a non-methylating host (26). Using the powerful selection with tetracycline resistance in this report it was unnecessary to take these precautions. At present we are introducing additional restriction sites into the vector which will require more refined technology.

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