A new phagemid vector for positive selection of recombinants based on a conditionally lethal barnase gene

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Abstract A new phagemid cloning vector for positive selection of recombinants, pBa-7, was constructed which contains an active barnase gene encoding the cytotoxic ribonuclease from *Bacillus amyloliquefaciens*, under control of the *lac* promoter. PBa-7 is a derivative of the high-copy number pBluescript II KS+ phagemid in which the modified barnase killer gene has been fused downstream from the *lac* promoter of the pBluescript II KS+ multiple restriction site. When a *lac*I^q-negative *Escherichia coli* strain is transformed by this vector, the active barnase blocks bacterial growth by massive RNA destruction [1]. However, if barnase is inactivated by insertion of a foreign DNA fragment into the multirestriction site of the vector, this recombinant plasmid no longer interferes with the host viability. The positive selection of recombinant clones is highly efficient and bench manipulations are considerably simplified. When *E. coli* transformants are plated out on rich medium with ampicillin, only cells containing recombinant plasmids give rise to colonies. In a *lac*I^q-positive host, the positive selection is IPTG-dependent. Therefore, pBa-7 phagemid can be amplified and prepared in large quantities from *lac*I^q-positive *E. coli* hosts. Hence, pBa-7 seems to be suitable for most genetic engineering manipulations.
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Key words: Barnase; Barstar; Cloning; Ribonuclease

1. Introduction

Positive selection cloning vectors are efficient tools simplifying in vitro DNA recombination procedures. A variety of plasmid vectors for positive selection has been described. They rely on the inactivation of either a lethal gene [2–5], a lethal site [6], a dominant function conferring the cell sensitivity to metabolites [7,8] or a repressor of an antibiotic-resistance function [9]. However, most of these vectors are not well-adapted for general use due to their large size, the limited range of useful restriction sites and the need for specific host strains or culture medium.

Recently, we described a plasmid vector (pMT440) which utilizes the toxic effect of the bacterial ribonuclease barnase from *Bacillus amyloliquefaciens* for positive selection of recombinant clones [1]. In the presence of the co-expressed barnase inhibitor barstar, pMT440 may be carried in any *Escherichia coli* harboring the *lac*I^q gene. The vector is lethal, however, when the *tac* promoter in front of the barnase gene is induced by IPTG. For *lac*I^q-negative *E. coli* strains, pMT440 is lethal without induction. The multirestriction site (MRS) of pUC19 was inserted into the barnase gene in place of the Val-36 codon, a site which does not alter the lethal effects of barnase [1]. Hence, this pMT440 contained the barnase-barstar cassette, the entire pUC19 polylinker under the control of the *tac* promoter and a vector fragment containing the *ori* and the ampicillin-resistance gene of the pUC19 plasmid. Uncut or re-ligated pMT440 does not support growth, whereas bacteria survive when transformed by plasmids containing inserts in the barnase gene, since the insertion of a foreign sequence into the center of the barnase gene inactivates the enzyme.

However, pMT440 still has some disadvantages: (i) the MRS is not flanked for the annealing of universal sequencing primers and (ii) it not only lacks the *f1* origin, necessary for obtaining single-stranded DNA, but (iii) also lacks T3/T7 sites for in vitro transcription. These drawbacks could be overcome by the creation of a new vector using barnase as a conditionally lethal gene in connection with the pBluescript II KS+ phagemid vector. For the construction of pBa-7, we amplified the barnase gene with specific primers for the flanking *Sac*I sites and cloned it into pBluescript II KS+ again using *Sac*I sites. The barnase gene was thus placed directly behind the polylinker. Cloning of an insert into the polylinker of this new vector causes a frame shift and/or inactivation of the barnase gene. In the absence of the leader peptide sequence in the barnase gene and the barnase inhibitor barstar, the intracellular active barnase was raised to toxic concentrations which interfered with the plasmid amplification. A special mutated barnase gene with six additional amino acids in place of the GTG codon of Val-36, encoding an enzyme with a slightly reduced ribonuclease activity [10], was used to solve this problem.

The new phagemid vector pBa-7 not only offers the same possibilities as the pBluescript II KS+ vector for gene manipulations, but also has the advantage that only recombinant clones give rise to viable colonies which would otherwise be discriminated from non-recombinant ones with the blue white screening technique [11,12], thus allowing direct positive selection.

2. Materials and methods

2.1. Construction of the pBa-7 phagemid vector

Plasmid pBa-7 was constructed as follows: *Sac*I sites were created by PCR on both sides of the barnase-barstar cassette. Amplification was carried out with plasmid pMT438a [10] and with two oligonucleotide primers, 5′-TGTGACAGAGCCGAAGTTCAACACGTT and 5′-CATCTCCGAGTCTCGTTTAAGAAAGTATGATGGT. The *Sac*I-digested PCR product was cloned into the *Sac*I site of pBluescript II KS+ using standard procedures and XL-1blue competent cells (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F′ proAB lacI^{q}ZDM15 Tn10 (Tetr)*) as described in [13]. The sequence of the 33 base oligonucleotide was chosen so that after elimination of the *Sac*II site in the MRS of the vector using *Sac*II and the Klenow fragment

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(Life Technologies, Karlsruhe, Germany), as described by [13], the barnase gene could be fused in frame downstream from the MRS of pBluescript II KS+. The recombinant construct consisting of the barnase gene fused with the encoding sequence of the 54 N-terminal amino acids of *lac*Z, the MRS of pBluescript II KS+ and barstar was designated pBa-2.0 (Fig. 1). Thereafter, a spontaneous PCR mutation in the barstar gene could be selected in which the sequence GAGCTT (position 837 in the sequence of the barnase gene: [14]) was transformed into the *Sac*I site G↓AGCTC. Using this *Sac*I site, a DNA fragment encoding amino acids 25–89 of the barstar gene was removed. In this vector, designated pBa-3s, only an inactive barstar peptide consisting of the first 24 N-terminal amino acids of the barstar is synthesized. A subsequent PCR amplification step with the two oligonucleotide probes 5′-TGTGACAGAGCTCGCACAGGTTATCAACACGTT and 5′-GACCGCGGGAGCTCACACTTTATGTAAAGCTGAA was performed for complete removal of the barstar sequence. The construct pBa-6s (not shown on Fig. 1) was obtained by exchange cloning the *Sac*I-digested PCR product into the *Sac*I cut pBa-3. The pBa-6 plasmid possesses two respective *Sma*I, *Eco*RI and *Hin*dIII sites, one in the MRS and the other in the central part of the barnase gene. In order to preserve only one unique *Sma*I, *Eco*RI and *Hin*dIII site in the MRS, the central *Sma*I, *Eco*RI and *Hin*dIII sites were modified by overlap extension PCR-directed mutagenesis as described [15], using the following two mutagenizing oligonucleotides for two rounds of site-directed mutagenesis, 5′-CTGCAAGGTTCCCTTTTGATGCTAAGCTCTTCTGAAATTCCC (for *Eco*RI and *Hin*dIII) and 5′-GCTTTTGCCCGGAAGTTTGCCTTCCC (for *Sma*I).

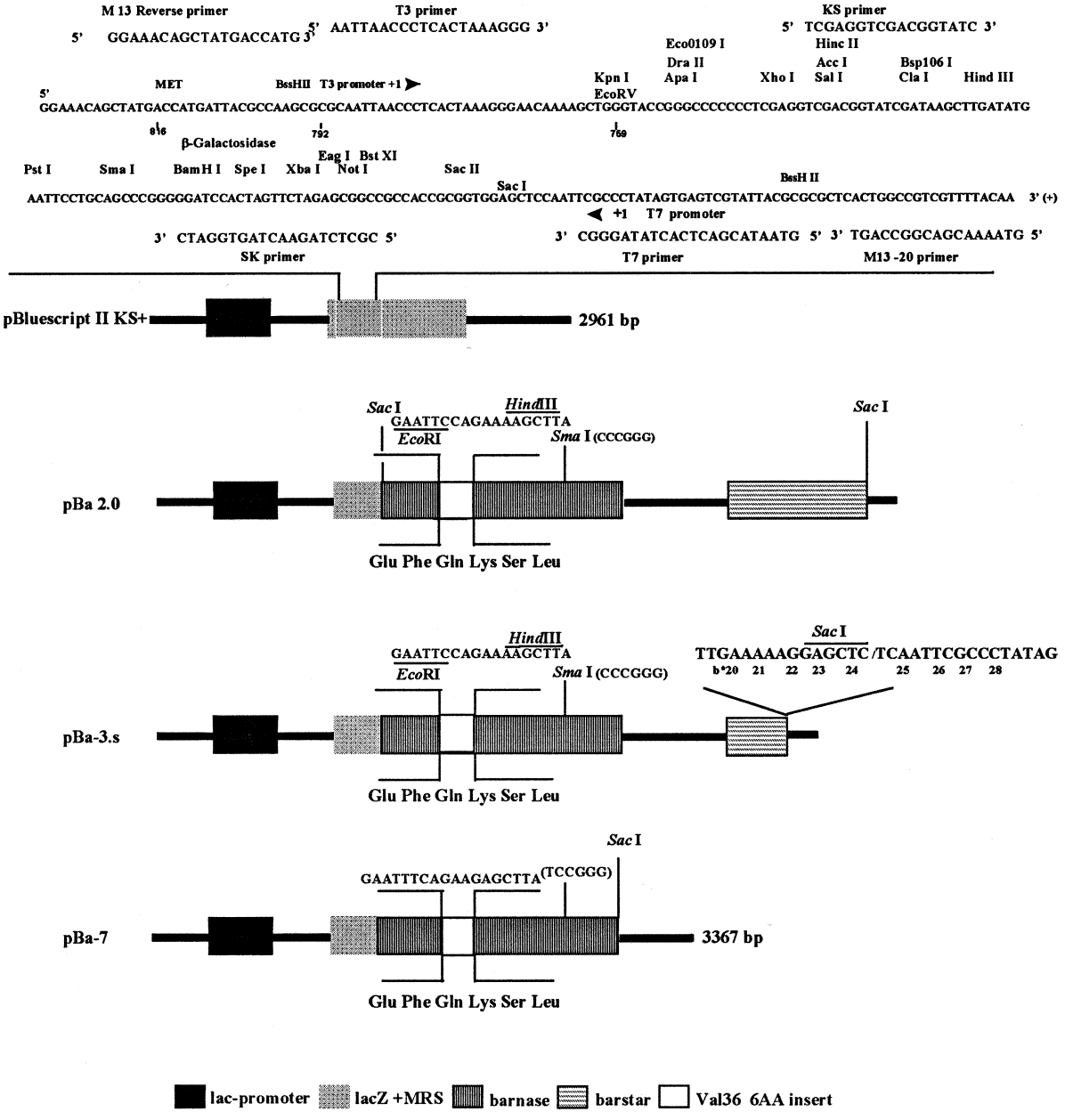

Fig. 1. Construction of the pBa-7 phagemid vector. The location of unique restriction sites in the polylinker, annealing sites of the universal primers, the *lac*P gene, the barnase gene and the barstar gene are shown. The ampicillin-resistance gene, the *Col*E1 origin of DNA replication and *f1* origin are not presented. DNA inserts may be sequenced with this pBa-7 vector by using a reverse M13 primer (GGAAACAGCTATGACCATG), T3 primer (AATTAACCCTCACTAAAGGG), KS primer (TCGAGGTCGACGGTATC) and SK primer (CGCTCTAGAACTAGTGGATC).

2.2. Positive selection cloning with the pBa-7

Total cellular RNA from Karpas 299 cells was extracted using a single-step isolation technique with Trizol Reagent (Life Technologies, Karlsruhe, Germany). The cDNA for PCR was synthesized using the Superscript Preamplification System with oligo$(dT)_{12-18}$ primers (Life Technologies, Karlsruhe, Germany). Subsequent PCR was performed with the *Bam*HI site-flanked human MDC-9-specific primers, 5′-CGCGGATCCTTGCTGTCTTGCCACAGACCCGGTATGTGGAG and 5′-CGCGGATCCTCCGTCCCTCAATGCAGTATTCATTTCATTGTATGT. The 1500 bp PCR fragment was extracted from agarose gels and ligated into *Bam*HI-digested pBa-7 utilizing components of the Sure Clone Ligation kit (Pharmacia Biotech, Freiburg, Germany). Competent TOP10 *E. coli* cells (*F- mcrA delta(mrr-hsdRMS-mcrBC) phi-80 lacZ-delta-M15 delta-lacX74 deoR recA1araD139 delta-(ara-leu)7679 galU galK rpsL(StrR) endA1 nupG*) from the One Shot Competent cells kit (Invitrogen, San Diego, CA, USA) were transformed by using a ligase reaction mixture, plated out and incubated for 14–16 h on LBA plates as described in the manufacturer's instructions. Recombinant clones were grown in LBA medium overnight followed by plasmid DNA extraction with the Qiagen Miniprep Plasmid Isolation kit (Qiagen, Hilden, Germany). Restriction analysis with *Bam*HI (Life Technologies, Karlsruhe, Germany) and colony PCR were performed as described in [13].

2.3. DNA sequencing

All nucleotide sequences were determined by the dideoxy method, using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster city, CA, USA). Subsequent separation and analysis were performed on a model ABI PRIZM 310 sequencer (Applied Biosystems, Foster city, CA, USA).

3. Results and discussion

As the basis for the construction of the positive selection vector pBa-7, which relies on the lethal effect of barnase, we choose the phagemid pBluescript II KS+ (Stratagene, Heidelberg, Germany). The scheme for the construction of pBa-7 is presented in Fig. 1. Like most other advanced vectors, the pBa-7 vector contains a large polylinker which is placed in front of the sequence of the barnase gene and is translated together with it. Hence, the polylinker adds 54 N-terminal amino acids to the recombinant barnase. The insertion of foreign DNA into the polylinker will lead to an inactivation of the barnase gene either by the induction of a frame shift or, when the inserts are of a sufficient size, by interruption of the barnase primary structure. The vector was obtained by cloning a 900 bp PCR fragment of the barnase-barstar cassette, flanked by *Sac*I sites, into the pBluescript II KS+ which also contains in its polylinker a *Sac*I site as the last restriction site. For a reduction of the cytotoxic effect of the barnase lacking the signal peptide, a mutant gene was used which contained the six inserted amino acids Glu-Phe-Gln-Lys-Ser-Leu instead of Val-36 [1,10]. The modified regions of this construct pBa-2 (Fig. 1) were sequenced and its toxicity for *lac*I^q-negative *E. coli* TOP10 cells was tested. However, pBa-2 turned out to be non-toxic and even the size of colonies was not reduced. This effect might possibly have been due to the 54 N-terminal amino acids of barnase representing the polylinker.

A spontaneous deletion mutant could be isolated in which a DNA fragment from the 3′-end of the barstar gene was omitted. Since this missing part of barstar is responsible for its inhibitory activity, the resulting construct, pBa-3s (Fig. 1), was strongly cytotoxic for the *E. coli* strain TOP10. This indicated that the barstar gene was not necessary in this variant of the vector design and it was therefore removed. Indeed, the resulting plasmid pBa-6 was reduced to 3367 bp and had a high lethal activity which is a prerequisite for the positive selection of recombinant clones. The pBa-6 vector was further improved by removal of the disturbing internal *Eco*RI, *Hin*dIII and *Sma*I restriction sites from the barnase gene by site-specific mutagenesis [15] and the resulting vector pBa-7 (Fig. 1) was tested for cloning of DNA fragments.

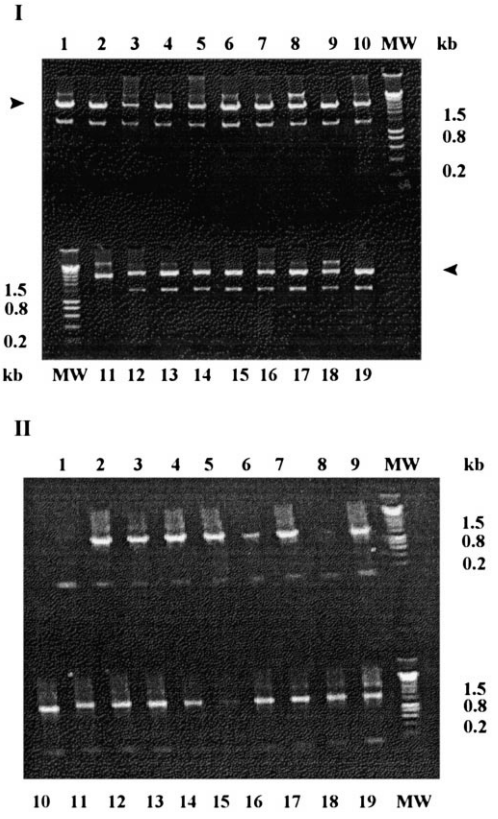

Fig. 2. Analysis of 38 individual recombinant inserts cloned using the positive selection vector pBa-7. (I) Lines 1–19 show *Bam*HI-digested DNA mini-preparations from *E. coli* TOP10 cells demonstrating that 18 of the 19 colonies contain a 1500 bp insert encoding the human reprolysin MDC-9. The arrows indicate the pBa-7 vector. (II) Lines 1–19 represent the analysis of bacterial colonies by direct PCR using KS and SK universal primers showing 18 out of the other 19 TOP10 recombinants containing an 1500 bp insert of human MDC-9.

The insertion of a foreign DNA fragment into the MRS of pBa-7 should lead to an inactivation of the barnase gene product. When TOP10 *E. coli* cells are transformed by ligation without induction by IPTG, recombinant plasmids should give rise to viable colonies, whereas the parental vector should be lethal for the host. A 1500 bp fragment of the reprolysin MDC-9 [16] was obtained by PCR amplification with specific primers which were flanked by *Bam*HI sites. These MDC-9 fragments were treated with *Bam*HI and ligated into the *Bam*HI-linearized pBa-7. After transformation of TOP10 *E. coli* cells with this construct and incubation on LB plates supplemented with 100 μg/ml ampicillin, 19 transformants were picked and their plasmid DNA was isolated. Restriction analysis with *Bam*HI showed that 18 out of 19 contained recombinant plasmids with inserts of the correct size (Fig. 2I). 19 Other transformants were analyzed with bacterial colony PCR using KS and SK universal primers (Fig. 2II). 18 Of these 19 colonies contained recombinant plasmids of the correct size.

We also used the pBa-7 vector for the cloning of a 800 bp

control PCR fragment from the Zero Blunt PCR Cloning kit (Invitrogen, San Diego, CA, USA) via the *Eco*RV and *Sma*I restriction sites. For all procedures such as ligation and transformation of the competent TOP10 *E. coli* cells (One Shot Competent *E. coli* kit: Invitrogen, San Diego, CA, USA), the original components of the kit were used according to the manufacturer's instructions. *Hin*dIII/*Bam*HI restriction analysis showed that all 20 picked clones obtained using the *Eco*RV site and that nine out of 10 transformants obtained by cloning into the *Sma*I site were positive and had an insert of the correct size. These results demonstrate that positive selection of recombinant clones using pBa-7 seems to function very efficiently.

The pBa-7 appears to be a suitable vector for general purpose cloning of DNA fragments. It seems likely that the same selection system could also be used in phages and perhaps in plasmids for different hosts. The applied selection strategy may be regarded as an example of the 'kill the rest' approach by taking advantage of charging vectors with destructive enzymes that ensure positive selection of the target clones.

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