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pTOC-KR: a positive selection cloning vector based on the ParE toxin

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Several prokaryotic cloning vectors have been developed to clone foreign DNA in bacteria. The insertion inactivation of β -galactosidase activity, for instance, is a common screening method of identifying recombinant DNA molecules in many vectors (1,2). However, this system poses several problems. For one, many vectors can self-ligate and give false transformants. Although β -galactosidase is widely used as a screening marker, it is limited to use with specially mutated *Escherichia coli* hosts for α -complementation. The color of the colonies is also hard to distinguish when the blue/white selection system is used. This system also

requires expensive reagents: 5-bromo-4-chloro-3-indolyl- β -D-galactosidase (X-Gal) and isopropylthio- β -D-thiogalactopyranoside (IPTG).

To address these problems, several positive selection vector systems have been developed based on the toxin-antitoxin systems parD (Kis/Kid) of plasmid R1 (3) and on the CcdAB system of plasmid F (4). Similar positive selection cloning vectors using the transcriptional factor GATA-1 (5) or a cellulase gene (*CelA*) as screening marker (6) have also been developed. Nonetheless, the application of these systems is also limited; in most cases, they are host-limited and their cloning

Figure 1. Map of pTOC-KR. The main feature of the vector is shown. Various multiple cloning sites and T7/T3 promoter sequences of the vector are represented.

efficiencies are not satisfactory.

This study developed a very efficient positive selection cloning vector based on the *parE* toxin gene derived from the *parDE* operon of the *Escherichia coli* plasmid RK2 (7,8). As the *ccdAB* system of plasmid F, the growth inhibition of ParE is induced by a postsegregational killing system (4). This system usually involves two proteins: one acts as a toxin, and the other as an antitoxin (7,9). By binding to the A subunit of DNA gyrase and restraining its activity (10), 12-kDa ParE inhibits the early stages of both chromosomal and plasmid DNA replication in *E. coli*. On the other hand, 9-kDa ParD binds to ParE to prevent its inhibitory activities and functions as an antitoxin (7,9,11).

To create multiple cloning sites (MCSs) in the internal region of the toxin gene, a stable region in the glutathione-S-transferase (*GST*)-*parE* gene was located through oligonucleotide insertion. Though several regions of the *GST-parE* gene such as *DdeI*, *EspI*, *AvaII*, *NgoMIV*, and *BclII* were used in this study, the *NgoMIV* site of the *parE* gene was only suitable for making MCSs. When a 36-mer synthetic oligonucleotide (with an unaltered open reading frame) was inserted into this region, ParE toxicity was still triggered. Likewise, *E. coli* containing this recombinant plasmid did not grow (data not shown). Nonetheless, other sites including *DdeI*, *EspI*, *AvaII*, and *BclII* were rendered unsuitable due to ParE destruction. The maximum length

of insert was determined to avoid toxin deactivation through MCS insertion. The size was 144 bp (data not shown), suggesting that longer insertions of cloning genes disrupt the ParE toxin effect.

A 3.56-kb positive selection cloning vector (pTOC-KR) was manufactured based on the *parE* toxin gene. The MCS of pTOC-KR contained various restriction enzyme sites such as *XbaI*, *KpnI*, *SacI*, *XhoI*, *PstI*, *SalI*, *SmaI*, *NotI*, *SphI*, and *HindIII* (Figure 1). T7 and T3 primer sequences were also included in the adjacent sites of the MCS. Digestion with restriction enzymes having specific corresponding sites within the MCS and DNA sequencing analysis confirmed the inserted MCS and T7/T3 primer sequences.

A serial toxin test was performed with the constructed pTOC-KR to examine the positive growth selection system. pTOC-KR was transformed into *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) with pRR46 plasmid, and several thousands of colonies were obtained from the LB-ampicillin + kanamycin plates (data not shown). From a colony of these transformants, pTOC-KR was purified and transformed back into *E. coli* XL1-blue. In the back transformation experiment, however, colonies did not grow well in ampicillin plates; only 2–3 colonies were observed per plate. The same results were obtained when these experiments were performed using other *E. coli* strains such as BL21, DH5 α , JM109, and JM110 (data not

shown). These results indicated that cell growth was still inhibited by ParE toxin activity. Such function is applicable to many other *E. coli* hosts because ParE toxin activity can function in various types of *E. coli* (12,13).

To test the cloning efficiency of pTOC-KR, various sizes of λ /HindIII-digested DNA fragments were cloned. Out of the randomly selected 50 colonies, 46 colonies contained one of the λ DNA fragments. Likewise, 4 out of 8 λ DNA fragments in these 46 clones were represented (Figure 2). No colony with a fragment longer than 4.3 kb was detected. The inability to clone large fragments of λ DNA might not reflect a constraint on the DNA fragment size, but it might suggest the expression of phage genes from the vector.

In another test, *HindIII*-digested 1-kb DNA fragments were cloned into a unique *HindIII* site located in pTOC-KR. The fragments were then ligated with the different ratios of vector to insert at 1:1, 3:1, 10:1, and 100:1. The following day, the proportion of colonies growing on the plates was greatly increased. Even when the ratio of vector to insert was 100:1, a large number of colonies grew on the plates (data not shown).

From each test, 50 colonies were randomly selected and subsequently amplified with the colony PCR to test for the presence of inserted fragments. About 48–49 colonies per sample group contained foreign DNA fragments. In addition, the restriction enzyme analysis revealed that all PCR-detected colonies contained the insert. These tests were performed several times, with at least 96% of the colonies always having desirable inserts despite the varying ratios of vector to insert to produce various numbers of colonies.

In conclusion, we have developed a positive cloning vector (pTOC-KR) based on the *parE* toxin gene. When pTOC-KR was transformed into *E. coli* without a foreign insert DNA, GST-ParE encoded from pTOC-KR completely prevented cell growth. When an insert DNA was cloned into the GST-ParE region of pTOC-KR, however, several thousands of transformants were obtained and at least 96% of all transformants had inserts. Moreover, dephosphorylation of pTOC-KR to

Figure 2. Cloning of λ DNA fragments by pTOC-KR. Fifty cloning samples were amplified to check for the presence of insert with PCR, and 20 samples are shown in this figure. Lane 1 is the λ /HindIII marker; lane 2, 11, 12, 18, and 19 are 2.0-kb λ DNA fragments; lane 3, 9, and 20 are 2.4-kb λ DNA fragments; lane 5, 6, 15, and 16 are 564-bp λ DNA fragments; lane 4, 8, 10, 13, 14, 17, and 21 are 125-bp λ DNA fragments; lane 22 is a negative control: only the multiple cloning site (MCS) region was amplified by T7 and T3 primer, and lane 7 did not contain any of the λ DNA fragments.

avoid self-ligation is not required, and transformants could be easily isolated without using X-Gal and IPTG. In addition, pTOC-KR had excellent cloning efficiency in various types of *E. coli* strains, such as *E. coli* XL1-blue, BL21, DH5 α , JM109, and JM110, and showed its potential broad host-range character in other types of *E. coli*.

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Cost-benefit analysis of a method using diatomaceous earth to purify Tamm-Horsfall protein

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Ever since Tamm and Horsfall first identified Tamm-Horsfall protein (THP) over a half-century before, its true function has baffled investigators (1). Studies have suggested that THP could have a role in prevention of urinary tract infection (UTI) by its adherence properties (2,3). THP is synthesized in the thick ascending kidney tubules and is not found anywhere else in the body, although the THP gene is evolutionarily conserved in all vertebrates (4). THP exists as a polymeric glycoprotein with a monomeric molecular weight (MW) of 88,000, and storage of THP has always proven to be difficult. THP quantitatively decreases over time even when dissolved in phosphate buffer at 4°C. This phenomenon was prevented when using Triton®X-100 and EDTA at an alkaline pH (TEA) instead of phosphate buffer (5).

The cost of commercial THP is \$155/100 µg (Biomedical Technologies, Stoughton, MA, USA). Currently, THP can be purified using two different published methods. The original method described by Tamm and Horsfall (1950) is time-consuming (1). The second method,

by Serafini-Cessi et al. (6), uses diatomaceous earth and then utilizes deionized water for the desorption of THP. This results in a reduced time of 4–6 h to purify the same amount of THP from urine, excluding dialysis and lyophilization.

Our aim was to utilize inexpensive and freely available materials to develop an uncomplicated and reproducible procedure with a shorter time frame. Molecular weight markers were obtained from Invitrogen (Carlsbad, CA, USA). The sodium dodecyl sulfate (SDS)-polyacrylamide precast gel (12%) was obtained from ISC BioExpress (Kaysville, UT, USA), and the filtering agent Celite® 521 and the diatomaceous earth (DE) were both from Sigma (St. Louis, MO, USA). DE was also obtained from Best Prices Storable Foods (Dallas, TX, USA). All other reagents were of analytical grade.

THP was purified using the most recent and quick method available (6).

Large volumes of 1.5 L of overnight urine were collected and neutralized with 10 M NaOH. It was then filtered through 20 g of DE layered on a Whatman no. 1