Loss of Tetracycline Resistant Gene from pBR322 by Direct Repeat Spontaneous Homologous Recombination

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Abstract

Objectives: It's well known that tetracycline resistant gene in pBR322 is about 1190bp clockwise in direction and tetracycline antibiotic inhibits bacterial protein synthesis. Resistant cells are able to grow in the presence of tetracycline because of efflux system. In this study, we try to delete tetracycline resistant gene from pBR322 by spontaneous homologous recombination between direct homology flanked regions .
Subject and Methods: There is direct repetitions Direct repeat between flank regions of tetrgene of pBR322 yield loss of tetrgene (Tetracycline Resistant Gene) by spontaneous homologous recombination. Restriction enzyme and T4 DNA ligation from NEB Phusion High Fidelity PCR Kit from NEB was used for PCR amplification. The measurement of rate of loss of tetrgene from pBR322 by homologous recombination was made using fluctuation test.
Results: After construction of new derivative from pBR322 which includes200bp flank region upper to tetrgene homology with downstream region in same direction, the measurement of rate of loss of tetrgene by fluctuation test was 64%.
Conclusion: The high rate of loss of tetrgene from pBR322 by direct repeat spontaneous homologous recombination indicated that homologous recombination between 200bp flank region for gene like tetrwhich it is about 1190bp consider as active method to delete the wanted gene.
Keywords: Fluctuation test, Tetracycline, Homologous recombination

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Introduction

The plasmid pBR322 was one of the first multipurpose cloning vectors to be available for efficient cloning and propagation of recombinant molecules in *E. coli*. This DNA molecule has been extensively used because of its simplicity and viability of its nucleotide sequence since the early days[(1)]. Today, pBR322 is still used as molecular cloning vehicle; although more advanced vectors have been developed from it.

The plasmid pBR322 is about 4361bp carries two sets of antibiotic which resistant genes ampicillin and tetracycline. Tetracycline antibiotics inhibit bacterial protein synthesis by preventing the binding of aminoacyl-tRNA molecules to the 30S ribosomal subunit[(2)].

The most clinically prevalent mechanism of tetracycline resistance is an active efflux system. Resistant cells are able to grow in the presence of tetracycline because a low intracellular concentration of drug is maintained. The agent responsible for resistance is a very hydrophobic membrane-associated protein which expels tetracycline against a concentration gradient, by coupling the efflux of a proton. The overall process is lectronutral, since tetracycline is cheated to a divalent cation when expelled [(3)].

Homologous recombination is a subject of major interest in biology. In vivo, this mechanism is involved in DNA repair mechanism and in the generation of genetic diversity [(4,5)].

Understanding and controlling homologous recombination is also of the utmost importance for medical and biotechnological purposes because of its implication and mutagenesis[6,7]. The main actors of homologous recombination are family of highly conserved proteins among which are RecA for *E. coli* and Rad51 for higher eukaryotes[8,9].

The three-strand reaction is the paradigm of homologous recombination. Its consider to feature in the early steps of the invivo process. It consists of (i) the ploymerization of RecA on a single stranded DNA (ssDNA) to form a nucleoprotein filament; (ii) the search for homology between the filament and neighboringdouple stranded DNA molecule (dsDNA); (iii) the alignment and paring of filament (containing the invading strand) with a homologous dsDNA (formation of a synapsis); and (iv) the exchange of strands, resulting in formation of heteroduplex (where in the invading strand is paired with its complementary strand) and in the ejection of the displaced strand[4].

2. Materials and Methods

2.1. Bacterial strains and plasmids

Escherichia coli K-12 W3110 (wild type) and DH5α (*sup*E44 D*lac*U169 [ϕ80 *lac*ZDM15] *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *rel*A1) were served as plasmid host strain. The plasmids include pBR322 (*bla*^{r}, *tet*^{r}), pBR-dRek(A5)(*bla*^{r},*tet*^{r}, *double Rek-sequence*), pBR-ΔTet (*bla*^{r}, *tet*^{s}). Isolation of plasmid DNA done by GenElute plasmid miniprep kit (Sigma Aldrich) while the purification was done by QIAquik purification kit (QIAGEN). Electrophoresis of DNA was carried out in 1% agarose slab gels[10].

2.2. Media and growth conditions

Liquid and solid media were based on LB medium (1%Bactotrypton, 0.5% yeast extract, 1% NaCl, 0.2% glucose and 1.6% Agar). Antibiotics were added to the medium when required to give the following final concentrations: ampicillin 200mg/L, tetracycline 25mg/L[11].

2.3. DNA manipulations and Transformation

Restriction enzymes and T4DNA ligase (New England Biolabs) were used for restriction/ligation of DNA. Cloning and transformation done according to conventional methods[10]. Fluctuation test was performed according to Lurie and Delbruck (1943)[12] method.

2.4. Fluctuation test

The fluctuation test devised by Lurie and Delbruck (1943)[12] allows the measurement of the rate of spontaneous homologous recombination between direct repeatRek sequences. An overnight culture was growing in 2ml LB medium at 37^{o}C with aeration, and dilutions to 10^{-6} were made in 0.9% NaCl. 100µl of diluted culture was used to inoculate each series of 10 tubes of 1ml LB plus glucose. Cells were grow overnight at 37^{o}C with vigorous shaking, resulting typical culture with 10^9cells/ml density. Overnight culture centrifuged, the cells resuspended in 100µl of 0.9% NaCl then diluted to 10^{-6} and 100µl plated on ampicillin plate. Overnight plate counted then replica plated on tetracycline-LB plate (contain 35µg/ml antibiotic) and ampicillin-LB plate (200µg/ml) then the results documented. As control pBR322 was used. The density of the cultures was measured by spectrophotometer at 600nm and by Macfrland tube no. 3 which give 10^9density[13].

Four experiments were done by fluctuation test to measure spontaneous recombination between doupleRek fragments to loss tetracycline resistance gene in pBR322-dRek plasmid, each experiment includes 20plates with other 20plates as control (pBR322).

2.5 Identification of Tetracycline Deletion

After overnight incubation, the colonies were tested for loss of *tet*^{r}gene by replica plating technique on tetracycline plate. Several colonies from each plate were checked by PCR amplification by two primers AMpus (CTGTCGGGCCCAGTTTACTCATATATAC) and PSGS1 (GTATCACGGGGCCCTTTCGTCTTCAAGA), and by restriction digestion by *EcoR*Iin compare to pBR322 as control.

3. Results

3.1. Construction of pBR322-dRek (A5) plasmid with two direct repeats (Rek) for homologous recombination.

A 150bp long sequence downstream of the tetracycline-resistance gene, was PCR amplified.

Primers Rek1 (TGAATGGAATTCGGCGGCAC) and Rek3 (CAGGACCGAATTCTGCCCGA), complementary to regions downstream of the *tet*^{r} gene, were used for this purpose. The obtained sequence was then cloned into the *EcoR*I-site upstream of the *tet*^{r}gene on plasmid pBR322, giving rise to plasmid pBR322-dRek (A5). In this plasmid the *tet*^{r} gene is thus flanked by two identical sequences in same orientation. These identical sequences, here after referred to as Rek-sequence (Fig.1), will serve as homologous recombination in order to delete *tet*^{r} gene from pBR322.

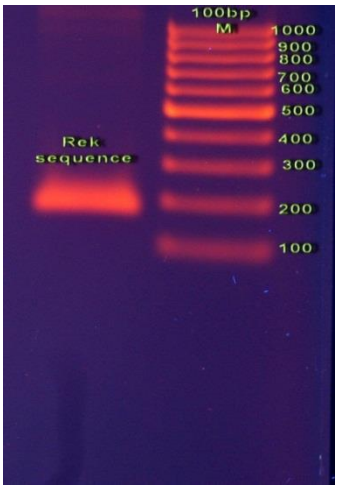

Figure 1: Shows PCR amplification of Rek sequence by two primers Rek1 and Rek3.

3.2. Detection of direction of Rek-sequence in pBR322-dRek (A5)

3.2.1. Detection of direction by restriction enzymes

Two restriction enzymes were used for detection of direction of Rek-sequence *Ava*I and *Hind*III. Rek-sequence in the same direction gave two bands by gel electrophoresis while antiparallel direction gave three bands when cut by *Ava* I and *Hind* III (Fig.2).

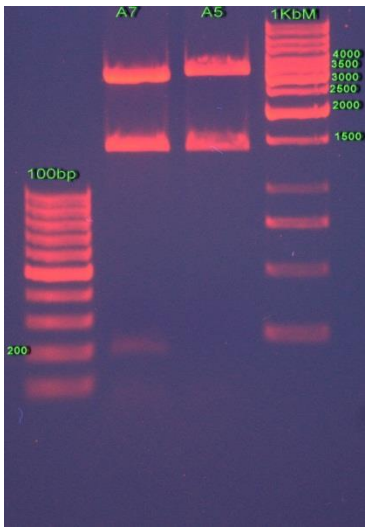

Figure 2: Shows restriction digestion by two endonuclease enzyme*Ava*I and *Hind*III. The plasmid pBR322-dRek (A5) gave two bands one about 1500bp other about 3200bp (same direction), while antiparallel Rek sequence gave thee bands (A7) 200bp, 1500bp, and 3000bp.

3.2.2. Detection of Direction by PCR amplification

For checking the direction of Rek-sequence in pBR322-dRek (A5) plasmid PCR amplification was done by the two primers Rek1 and Rek3. The result of amplification showed that the two Rek-sequence in the same direction (Fig.3).

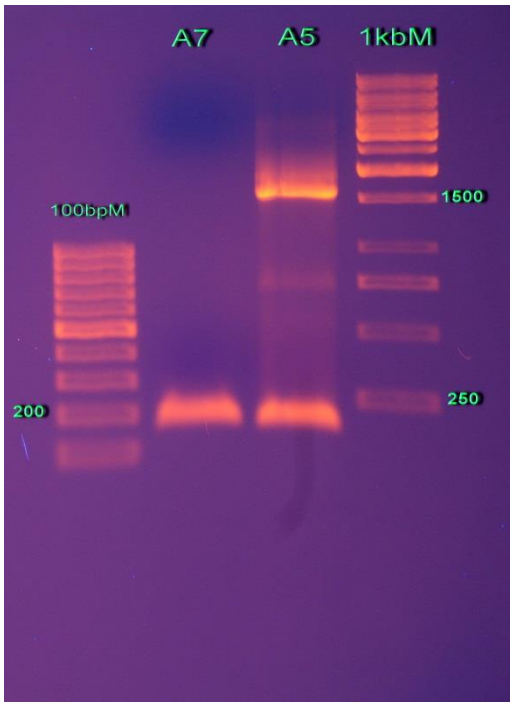

Figure 3: shows the detecting of direction of Rek sequence by PCR amplification by use Rek1 and Rek3 primers. The same direction sequence gave two bands (A5) while antiparallel sequence gave one band (A7).

3.3. Measurement of rate of Homologous Recombination

The rate of spontaneous homologous recombination between direct repeat of Rek-sequence in pBR322-dRek was measured by Lurie and Delbruck method[12]. Our results showed that the mean of growth cells on twenty (20) plates of tetracycline was 34 cells from 96 cells (Experiment (1)) in compare with amp plate for same cell origin which gave growth of 96 cells from 96 cells. Also the other three experiments showed similar results experiment (2) 34/95, experiment (3) 33/96, and experiment (4) 34/95 (Fig. 4).

The percentage of the rate of spontaneous homologous recombination between double Rek fragments and loss of *tet*^{r} gene was 65% in experiment (1), 64% in experiment (2), 66% in experiment (3), and 65% in experiment (4) (Fig. 5).

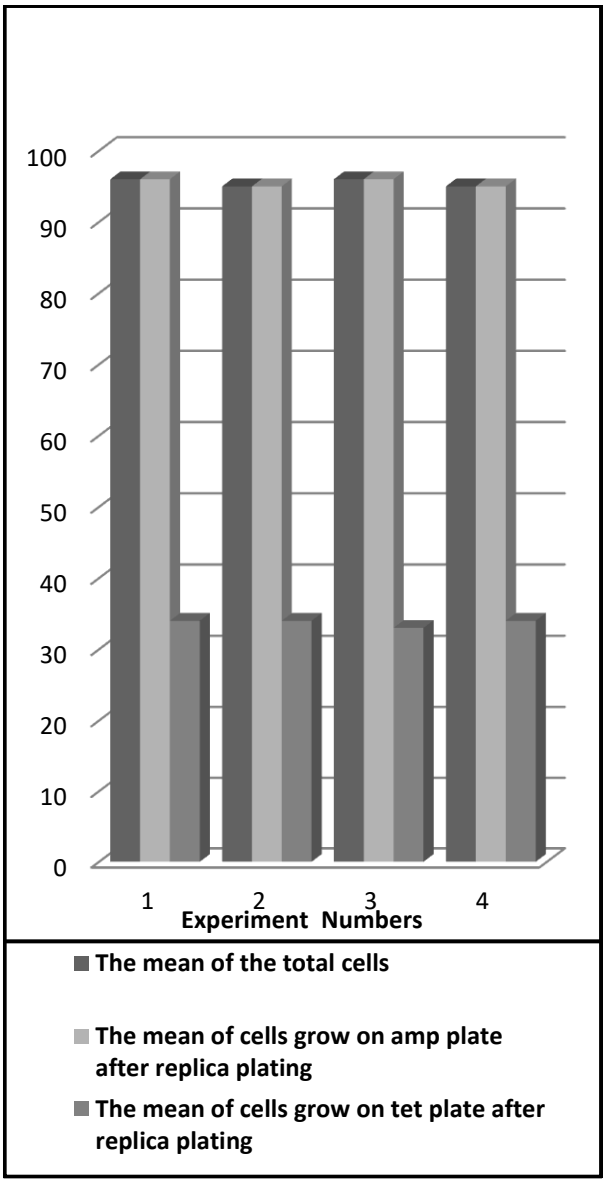

Figure 4: Shows the mean of the growing cells on amp and tet plate after replica plating in compare with mean of total cells for each four experiment.

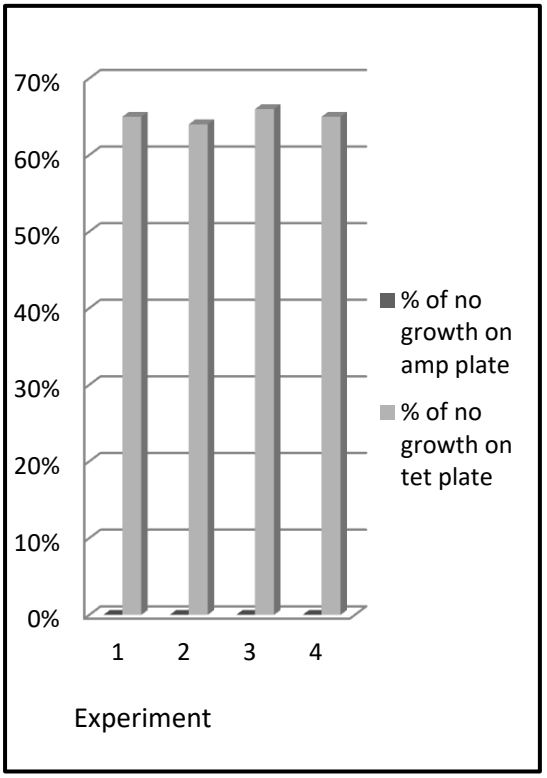

Figure 5: Shows the percentage of no growing cells on ampicillin (amp) and tetracycline (tet) plate .

3.4. Controls and Cell Viability

Two controls were used in this study. The plasmid pBR322 without any modification was used to ensure that was no loss of *tet*^{r} gene without double Rek sequence. Other control was pBR322-dRek (A5) on amp plate which is considered as viable control to confirm that the origin number of homologous recombination came from the number of cells which were grown on amp plate.

3.5. Confirmation of Deletion of tetracycline resistant gene

After replica plating on tetracycline plate and detecting the cells which had no growth, several colonies picked up and confirmed for loss of *tet*^{r} gene by PCR amplification by two primers AMpus and PSGS1 in comparision with pBR322 as control (Fig. 6). Restriction digestion was also used to confirm deletion of *tet*^{r} gene by endonuclease enzyme *Eco*RI(Fig. 7).

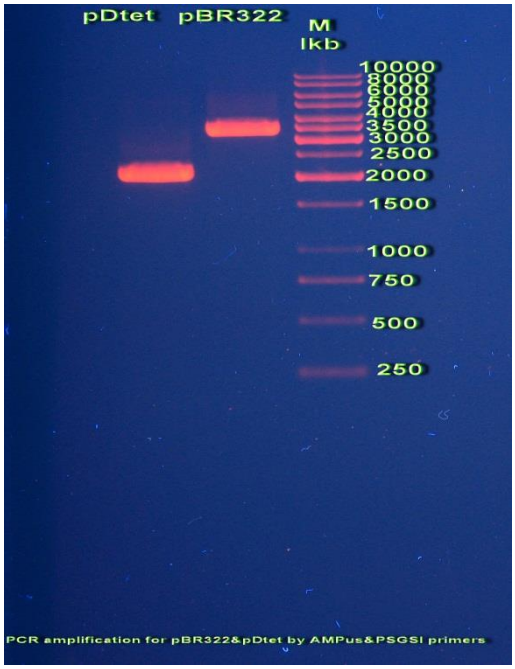

Figure 6: Shows the PCR amplification by two primers AMpus and PSGS1 to confirm the deletion of *tet*^{r} gene from pBR322-drek (pDtet) by spontaneous homologous recombination in compaire with pBR322 as control.

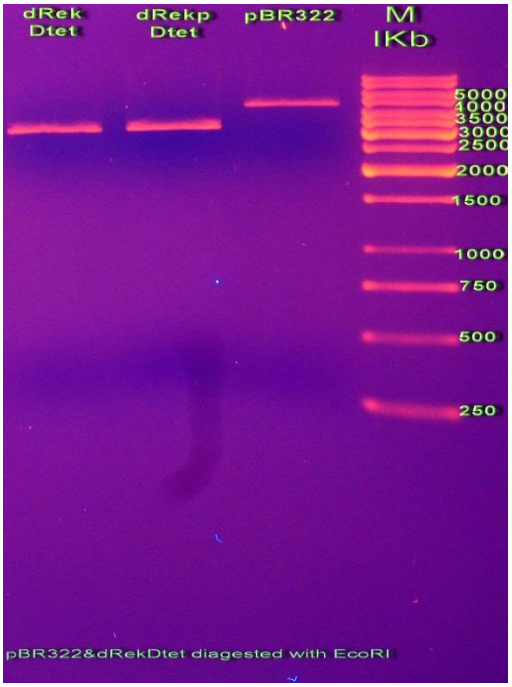

Figure 7: shows the restriction digestion by *Eco*RI enzyme to confirm the deletion of *tet*^{r}gene in compare with pBR322 as control.

Discussion

Homologous recombination is a type of genetic recombination in which nucleotide sequences are exchanged between two similar or identical molecules of DNA. It is most widely used by cells to accurately repair harmful breaks that occur on both strands of DNA. Homologous recombination also produces new combinations of DNA sequences. These new combinations of DNA represent genetic variation. Homologous recombination is used in horizontal gene transfer to exchange genetic material between different strains and species of bacteria and viruses[14].

We successfully amplified and cloned homology sequences named Req-sequence upstream to *tet*^{r} gene in pBR322 plasmid. The Rek-sequence was about 150bp and it homology to downstream region of *tet*^{r} gene in pBR322. The length of homology sequence is critical point to yield homology recombination. We found in our study that 150bp increase the yield of spontaneous homologous recombination and this was supported by other study. Napieralas and co-workers found that increasing the length of homologous sequences from 17bp to 165bp, 60 fold increase the recombination frequency between direct repeat[15]. Because the sequence homology is such an important feature of recombination, several investigators have studied the effects of decreasing the length of homology available for specific recombination events. Singer and colleges (1982)[16] determined that minimum of 50bp of homology is required by major homologous recombination pathway of bacteriophageT4. More recently, Gohda and Radding (1983)[17] found that E coli RecA protein efficiently paried molecules in which homology was limited to 30bp. Rubnitz and Subramani (1984)[18] documented in their results that high recombination frequency occurred between 163bp-214bp, with lower levels of recombination occurred when there was only 14bp of homology.

It was important to detect the direction of cloned fragment (Rek-sequence) because there was two probability of cloning result I) cloned fragment may be in the same direction with downstream Rek-sequence II) or antiparallel direction with downstream region. Deletion of any gene by homologous recombination requires homology sequence flank region in same orientation, while antiparallel orientation gives inversion gene[19]. This also supported by other study, Napieralas and co-worker (2002)[15] showed that the predominant products of recombination between directly repeated CTG.CAG tracts are intracellular deletions. The same researchers showed that the products of intracellular deletion between homology sequences have never been detected for plasmids harbouring inverted repeats.

Our result showed high rate of loss of tetracycline resistance gene from pBR322 by homologous recombination 64%(the average of 4 experiments). This is due to I) the size of flanked regions sequence, they were about 150bp which consider as best size of DNA which can give homologous recombination[16]. II) The flanked region is considered as non-translated region and that enhance the homologous recombination[20]. III) The plasmid pBR322 contains two selective markers (*bla*^{r},*tet*^{r}), when we tried to delete *tet*^{r} gene by homologous recombination, we left bla gene intact, which supports the plasmid to remain stable inside the cells.

The deletion of *tet*^{r} gene from pBR322 by direct repeat spontaneous homologous recombination is supported by Lurie and Delbruck theory (1943)[12], they observed that the occurrence of spontaneous mutations would result in much greater fluctuations in the observed number of Ton^r colonies per culture than random induction of resistance following contact with the phage (the induction) on assay plate. When we apply the experiment of Lurie and Delbruck to our experiment, we found that in the absence of induction, the spontaneous homologous recombination will occur (the induction here is tetracycline antibiotic) and lead to loss of tetracycline resistant gene (*tet*^{r}) from pBR322 plasmid. Hagg and co-workers (2004)[21]menshed that the plasmid born *tet*^{r} was deleted by taking advantage of *invivo*homologous recombination between two homologous regions of DNA flanking the *tet*^{r} gene using fusaric acid plate that selects for *tet*^{s} cells. That means they didn't get spontaneous homologous recombination but they induct the deletion of *tet*^{r} gene by fusaric acid. Bochner (1980)[22] and Maloy (1981)[23] used simple technique that allows direct plate selection of tetracycline-sensitive clones from predominantly tetracycline resistant population. The technique is based on their finding that tetracycline-resistant cells are hypersensitive to lipophilic chelating agents, such as fusaricscid. This finding supports the contention that certain metal ions critically facilitate tetracycline uptake and leads us to suggest possible molecular mechanisms for tetracycline resistance. Since tetracycline resistance is an inducible trait[24,25], chlortetracycline could be used to generate the membrane modifications associated with tetracycline resistance. When chlortetracycline is autoclaved in broth, it is denatured so as to lose its toxisity toward *tet*^{s} cells while retaining its inducing ability for *tet*^{r}cells[26].

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