Short Technical Reports

Novel Kanamycin/Neomycin Phosphotransferase Cassette Increases Transformation Efficiency in *E. coli*

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ABSTRACT

Stable transformation depends on the efficient delivery of DNA into cells and the robust expression of genes that encode proteins which provide resistance to selective (cytotoxic) compounds. We have examined the possibility that altering the 5¢ untranslated region (UTR) of a selectable marker may increase transformation efficiency. A 15-nucleotide synthetic UTR (the so-called universal translational enhancer [UTE]) was placed upstream of a kanamycin/neomycin phosphotransferase (kanaR) gene to create a novel expression cassette, UTE-kanaR. In comparison to a wild-type version of kanaR, UTE-kanaR produced up to 30-fold more transformants in E. coli*. The superior performance of UTE-kanaR was independent of the promoter strength, indicating that the gene may find general use in routine transformation experiments.*

INTRODUCTION

Stable transformation of cells is an essential tool in the arsenal of molecular biologists. Recombinant cells are selected by subjecting typically with the use of cytotoxic drugs (e.g., kanamycin) a population of transfectants to conditions that eventually kill wild-type (WT) cells. Fundamental to the selection process is the ability of transfected cells to express a gene that encodes a protein or an enzyme that binds to or metabolizes the cytotoxic (selection) compound. From these considerations, it appears that success at stable transfection is a function of two key events: first, the efficiency with which DNA is delivered into the appropriate intracellular locations and second, robust expression of the gene that encodes the selectable marker.

Attempts at increasing transformation efficiency have focused solely on the development of tools (e.g., electroporators and ballistic guns) aimed at increasing the ability of DNA to traverse biological membranes. Little attention has been paid to the manipulation of control regions of genes that encode selectable markers as a mean of enhancing transformation efficiency. In this work, we tested the possibility of raising transformation efficiency by altering the 5′ untranslated region (UTR) of a kanamycin/neomycin phosphotransferase (kanaR) gene (1).

A recently described UTR, the universal translational enhancer (UTE) (10), was juxtaposed with kanaR, creating a novel kanaR cassette, UTE-kanaR. UTE-kanaR plasmids produced up to 30-fold more transformants in *Escherichia coli* than did those containing a wild-type kanaR. The possible implications of these observations are discussed.

MATERIALS AND METHODS

Strains

In this study, we used *E. coli* DH5α. All bacterial cultures were performed in Luria-Bertani (LB) medium at 37°C.

Vectors

pXUTE-kanaR. Primers UTE-Kana-F (forward primer) (5′-TAAGAACGTTTTCCATAACTTAGGAGGCAGATCATGATTGAACAAGATGGA-3′) and Kana-rev (reverse primer) (5′-TATGAACGTTTTCTTAGAAGAACTCGTCAAGAAG-3′) were used to amplify a 0.8 kb coding region of the kanaR gene (5). AmpliTaq® DNA Polymerase (PE Biosystems, Foster City, CA, USA) was used with pHSS6 (8) as template. The inclusion of *Xmn*I sites at the termini of the primers (UTE-Kana-F and Kana-rev) (Figure 1A) facilitated insertion into the *Sma*I site of pX63HYG (3) and generated pXUTE-kanaR. (All restriction enzymes were from New England Biolab, Beverly, MA, USA).

pXWT-kanaR. To obtain a WT 5′ UTR (i.e., from Tn5) for kanaR forward primer (WT-Kana-F) (5′-TAA**AGATCT**ATGAGGATCGTTTCGCATG-3′) was used in combination with Kana-rev in amplification. A primer encoded *Bgl*II site is in bold. The amplified PCR fragment includes the coding region plus 20 bases upstream of the translational initiation (ATG) codon. The fragment was cloned into a *Bgl*II-*Sma*I site of pX63HYG (3), generating pXWT-kanaR.

Subcloning of kanaR into pBluescript® II. Appropriate *Bgl*II-*Nsi*I fragments were excised from either pXUTE-kanaR or pXWT-kanaR (Figure 1A), and ligated into the *Bam*HI–*Pst*I site of pBluescript II. The recombinant plasmids were named pBSUTE-kanaR and pBSWT-kanaR, respectively.

Measurement of Transformation Efficiency

Twenty nanograms of plasmid DNA purified by the QIAGEN®-tip 500 (Qiagen, Valencia, CA, USA) was used to transform *E. coli* by a $CaCl_2$ protocol (7). Cells were diluted with LB broth and plated on LB/kanamycin (30 µg/mL) plates. Plating was also done on LB/ampicillin plates (100 µg/mL) as a control. After overnight incubation at 37°C, colonies of transformants were counted.

Growth Assay

Transformants from LB/ampicillin plates were used for the growth assay. Three colonies of each type were resuspended in LB medium. The initial cell density was measured (OD_{590}). An equal number of cells were then inoculated into 10 mL of LB medium supplemented with kanamycin or ampicillin. The culture was incubated at 37°C with shaking at 240 RPM. OD_{590} readings were obtained periodically.

RESULTS

UTE-kanaR: A Novel Cassette Encoding Resistance to Kanamycin

Two 5′ UTRs, the WT kanaR 5′ UTR (WT-kanaR) and a kanaR containing a UTE (10) (see Materials and Methods for details), were linked to kanaR to examine their influence on transformation efficiency. The resulting

kanaR genes, termed WT-kanaR and UTE-kanaR, respectively, were cloned into pX63Hygro, which contains the genes that encode resistance to hygromycin and ampicillin. The derivative plasmids were named pXWT-kanaR, containing the WT-kanaR and pXUTE-kanaR containing the UTE-kanaR gene (Figure 1A). A map of pXWT-kanaR is not provided; the only difference between it and pXUTE-kanaR is the sequence immediately upstream of the kanaR gene, as provided in Figure 1A. The reader's attention is drawn to the availability of three genes for antibiotic resistance on both test plasmids, two of which, β-lactamase (ampR) and kanaR, are expected to be useful in *E. coli*.

pXUTE-kanaR Increases Transformation Efficiency

We tested the effect of the two 5′ UTRs on transformation efficiency by transfecting *E. coli* with pXUTE-kanaR (Figure 1) or pXWT-kanaR. Using 20 ng of pXUTE-kanaR, we obtained 9500 colonies, while pXWT-kanaR produced only 300 transformants (Figure 1B). This represents a 30-fold difference in transformation efficiency between the two kanaR constructs. To check whether the use of a large amount of DNA could eliminate the difference in transformation efficiency, we used 200 ng of each plasmid. Here, pXUTE-kanaR yielded 64 000 colonies while pXWT-kanaR produced 3800 colonies. Hence, the difference observed in transformation efficiency is within the same order of magnitude as that obtained with smaller amounts of DNA.

In control experiments, we selected on ampicillin plates a portion of the mixture of cells that were spread on kanamycin plates. Attesting to the fact that equivalent amounts of DNA were used in the kanamycin experiments, both pXWT-kanaR and pXUTE-kanaR produced approximately the same number of transformants: 34 000 and 32 000 colonies, respectively. This result is expected because the ampR gene on both

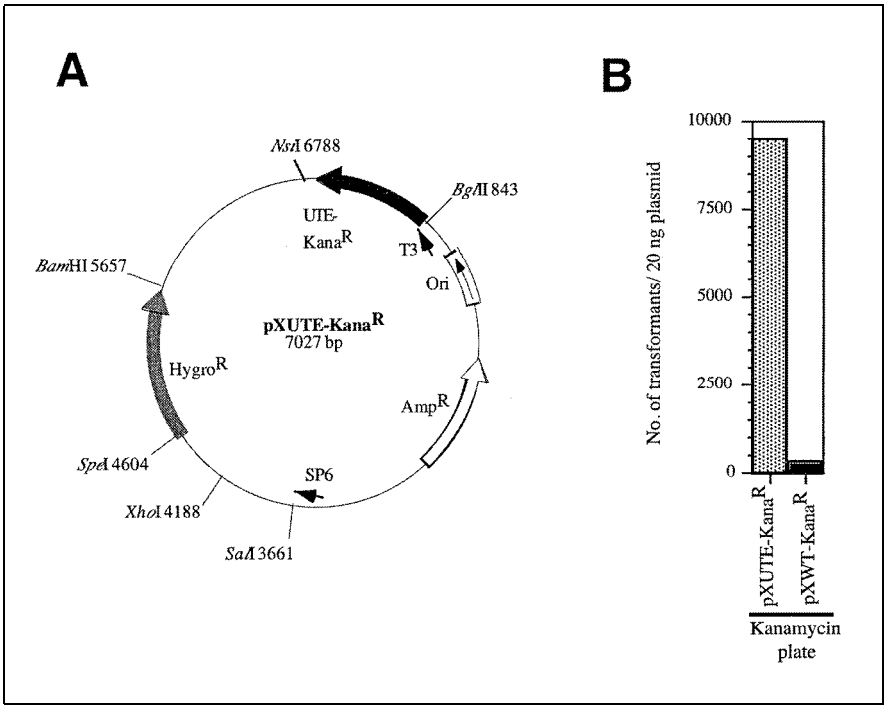

Figure 1. (A) Graphic map of the pXUTE-kanaR. The Hygro^R gene is flanked by the 5′-upstream and 3′-downstream regions of the *Leishmania* DHFR-TS (dihydrofolate reductase-thymidylate synthase) gene. Location and orientation of T3 and SP6 promoters are shown by arrows. (B) Efficiency of pXUTE-kanaR compared to pXWT-kana^R: transformation on kanamycin plates. Plasmids were transfected into *E. coli* DH5α (see Materials and Methods). After allowing the cells to grow in LB broth (600 μL) at 37°C for 1 h, they were diluted with LB medium (pre-warmed at 37°C) and plated on LB/kanamycin plates. Numbers are taken from the average of two plates.

plasmids is identical. With 200 ng of DNA, both plasmids yielded 130 000–140 000 colonies.

pXUTE-kanaR/*E. coli* Grow Faster in a Liquid Medium than pXWT-kanaR/*E. coli*

We were curious to know whether the effectiveness of pXUTE-kanaR was an artifact of colony formation on plates. If UTE-kanaR caused a fundamental alteration in the physiology of cells bearing the plasmid, we predicted that a phenotypic difference similar to that observed during colony formation would be manifested during the growth of pXUTE-kanaR/*E. coli* and pXWT-kanaR/*E. coli* in a liquid medium. We first tested these ideas by selecting transformants of pXUTE-kanaR and pXWT-kanaR on ampicillin plates. We then seeded cells (DH5α) from the ampicillin plates in a liquid medium containing kanamycin at 30 µg/mL (Figure 2A) or 200 µg/mL (Figure 2B), and we monitored the growth characteristics.

The doubling time of pXUTE-kanaR/DH5α in medium containing 30 µg/mL of kanamycin was 40 min (Figure 2A). In contrast, the cell division time of pXWT-kanaR/DH5α was 75 min (Figure 2A). Thus, pXUTE-kanaR enabled the bacterial cells to grow better in a liquid medium. In a medium containing 200 µg/mL of kanamycin, the differences in growth rate of the two strains of DH5α were exacerbated. While pXUTE-kanaR grew with a doubling time of 45 min, cells containing pXWT-kanaR failed to multiply (Figure 2B). These results parallel those obtained during selection of the transfected cells on kanamycin plates (Figure 1). We conclude that the difference in transformation efficiency between pXUTE-kanaR and pXWT-kanaR is not restricted to growth on plates.

We considered the possibility that the higher transformation efficiency and better growth rate of pXUTE-kanaR-containing *E. coli* was the result of higher plasmid copy number. Two lines of evidence weigh against the proposal. First, for an equal amount of DNA, the transformation efficiency of both pXUTE-kanaR and pXWT-kanaR was almost identical on an ampicillin plate. More importantly, a possible effect of kanamycin on the plasmid copy number was checked directly. For this purpose, pXWT-kanaR/DH5α and pXUTE-kanaR/DH5α cells were cultured for 2 h in 200 µg/mL kanamycin (Figure 2B). Episomal DNA was purified from the two strains. From 4 mL pXWT-kanaR/DH5α (2×10^7 /mL), 50 ng of DNA were obtained. From a milliliter of pXUTE-kanaR/DH5α (2×10^8/mL), the yield was 160 ng. From these data, we surmise that for each 4.9

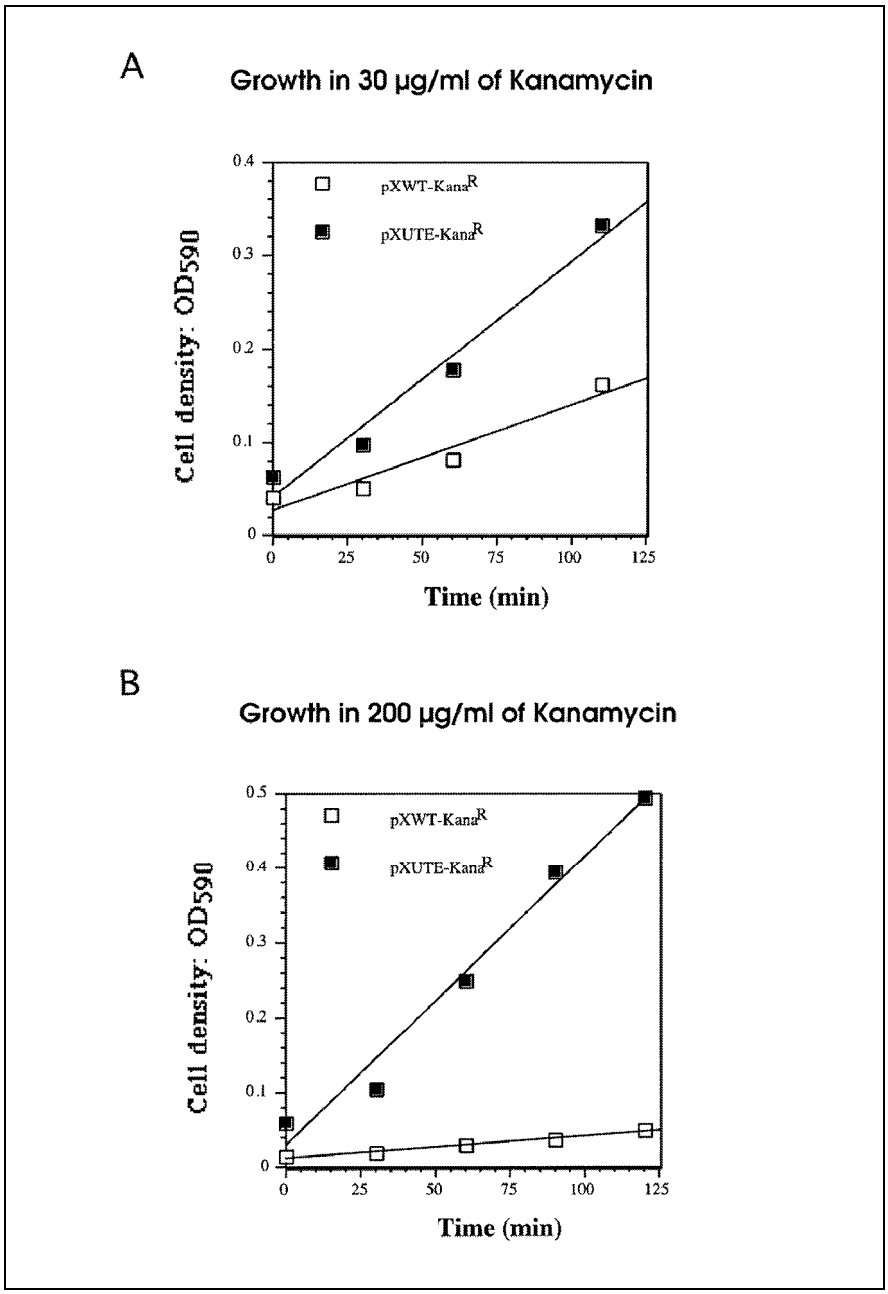

Figure 2. Growth of pXUTE-kanaR/DH5α and pXWT-kanaR/DH5α in kanamycin-containing medium. (A) An equal number of cells was diluted (final OD_{590} approximately 0.005) in LB supplemented with 30 µg/mL kanamycin. Cell growth was monitored spectrophotometrically at the indicated times beginning 2–3 h after dilution. Data presented is an average from duplicate cultures. (B) Two colonies of DH5α transformants harboring pXWT-kanaR or pXUTE-kanaR were cultured in LB (plus 25 µg/mL of ampicillin) at 37°C. Cultures were then diluted (>20-fold) into LB containing 200 µg/mL of kanamycin to an OD_{590} of approximately 0.005. Measurement of cell density was initiated after 2–3 h and continued at the stated intervals. Data shown is an average from a duplicate experiment.

$\times 10^6$ cells, the amount of plasmid DNA obtained was 4 and 3 ng, for pXUTE-kanaR/ DH5α and pXWT-kanaR/DH5α, respectively. Thus, in spite of a ten-fold difference in cell density at the 2 h time point (Figure 2B), the amount of DNA per cell was not different in the two strains. This conclusion was confirmed by transformation of *E. coli* with these DNA preparations and selection on LB/amp plates. The transformation efficiencies were 8.5×10^6/μg (pXUTE-kanaR) and 7.7×10^6/μg DNA (pXWT-kanaR).

A Strong Promoter Does Not Abolish Differences in Transformation Efficiency between pXUTE-kanaR and pXWT-kanaR

The plasmids used up to this point do not contain strong promoters upstream of the kanaR constructs. Presumably, transcripts for kanaR originate from cryptic promoters upstream of the genes. Alternatively, they could be run-throughs from transcription across *ori* (Figure 1A). These considerations raised the possibility that the use of a strong promoter might synthesize enough WT-kanaR RNA such that the difference in growth rates between pXWT-kanaR/DH5α and pXUTE-kanaR/DH5α might be lost. To test this hypothesis, the kanaR cassettes were cloned downstream of the β-galactosidase (*lac*Z) promoter in pBluescript II (see Materials and Methods). We compared these new pBluescript derivatives, pBSUTE-kanaR and pBSWT-kanaR (Figure 3A) on plates embedded with either ampicillin or kanamycin.

Transformation efficiency on ampicillin plates was equivalent for both pBSUTE-kanaR and pBSWT-kanaR (approximately 11 000 colonies per 20 μg of DNA). However, on kanamycin plates the UTE-kanaR construct was at least ten-fold better at generating colonies than the WT-kanaR gene (Figure 3B). Thus, UTE-kanaR functions better than WT-kanaR even when strong promoters drive transcription.

DISCUSSION

The ability of investigators to obtain large numbers of transformants after introduction of plasmids into cells can determine the success of many a cloning project. For example, in constructing genomic or cDNA libraries, it is critical to obtain a maximum number

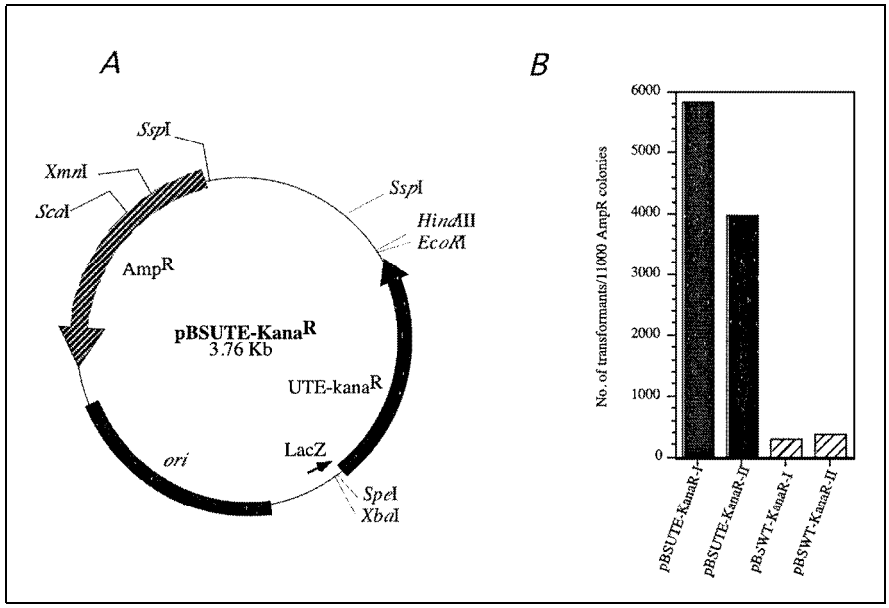

Figure 3. Effect of a strong promoter in influence of UTE on transformation efficiencies. (A) Graphic map of pBSUTE-kanaR showing the location and orientation of a *Lac*Z promoter upstream of Kana^R. (B) Depicts number of transformants per 20 ng of plasmid normalized for 11 000 ampicillin resistant colonies produced by the same amount of plasmid. pBSUTE-kanaR I and pBSUTE-kanaR II (black bars) represent two different clones. pBSWT-Kana^R I and II (open bars) are also different clones.

of clones. Theoretically, two important parameters are likely to contribute to transformation efficiency: first, the ease with which DNA is introduced into cells and second, the proficiency of expression of selectable markers.

With the advent of DNA transfection by electroporation and ballistic guns, those technologies have risen to the forefront of attempts to increase transfection efficiency. Nevertheless, it is clear that to achieve the highest transformation efficiency, it is advantageous to combine high transfection efficiency with outstanding conditions for expression of the drug resistance marker. Surprisingly, the latter area of investigation, which involves the application of basic principles of protein expression and intracellular targeting to selectable markers, has been ignored.

Translation in prokaryotes is facilitated by a Shine-Dalgarno sequence (UAAGGAGG), a ribosome binding site (RBS), positioned 4–11 nucleotides from the initiation codon (AUG) (2,6). UTE is a synthetic 5′ UTR consisting of the λ *cro* Shine-Dalgarno box (AGGAGG) (4) separated by a mutant *lac*Z spacer (CAGATC) from the translational initiation codon. The superiority of UTE over the wild-type UTR of kanaR could be the result of a combination of two factors. First, the RBS of WT-kanaR UTR (i.e., AGGA) is sub-optimal, while the RBS of UTE (AGGAGG) approaches the optimal sequence (10). Second, it was demonstrated recently that the -3 to -1 region of an initiation codon can influence protein synthesis in *E. coli*. Cytidines at the -3 and -1 positions are not favorable. Curiously, the -3 to -1 positions of WT-kanaR UTR are occupied by Cs. UTE-kanaR contains an A, which is favored at the -3 position (10).

Kanamycin is thought to act primarily by interfering with ribosomal protein synthesis, while ampicillin inhibits peptidoglycan synthesis. These differences in the mechanism of action of the antibiotics dictate that the efficiency of transformation will not be equivalent when different selectable markers are used (Figure 1). A related observation has been made earlier for tetracycline and ampicillin (9). Apparently, transformation with wild-type kanaR is less efficient by two orders of magnitude than transformation with ampR (see Results section), a fact that might have contributed to the widespread use of ampR in cloning vectors. With the introduction of UTE-kanaR, transformation efficiencies of the two selectable markers are now within the same range, although ampR has the upper hand by a factor of three.

Our effort with UTE is the first attempt to increase transformation efficiency by altering the noncoding region of the gene for a selectable marker. We chose kanaR for these initial studies because UTE-kanaR can be used to construct a single cloning/expression vector that provides resistance to kanamycin in prokaryotes (and possibly plants) and possibly to G418 in eukaryotes.

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Localized Electroporation: A Method for Targeting Expression of Genes in Avian Embryos

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ABSTRACT

Avian embryos are a popular model for cell and developmental biologists. However, analysis of gene function in living embryos has been hampered by difficulties in targeting the expression of exogenous genes. We have developed a method for localized electroporation that overcomes some of the limitations of current techniques. We use a double-barreled suction electrode, backfilled with a solution containing a plasmid-encoding green fluorescent protein (GFP) and a neurophysiological stimulator to electroporate small populations of cells in living embryos. As many as 600 cells express GFP 24–48 h after electroporation. The number of cells that express GFP depends on the number of trains, the pulse frequency and the voltage. Surface epithelial cells and cells deep to the point of electroporation express GFP. No deformities result from elec-