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Cloning of the *npt*II gene of *Escherichia coli* and construction of a recombinant strain harboring functional *rec*A and *npt*II antibiotic resistance

S. Ghanem

Botany and Microbiology Department, Faculty of Science,
Helwan University, Ain Helwan, Cairo, Egypt

Corresponding author: S. Ghanem
E-mail: samah.ghanem@laposte.net

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ABSTRACT. In an attempt to clone the ORF of the *npt*II gene of *Escherichia coli* K12 (ATCC 10798), two degenerate primers were designed based on the *npt*II sequence of its Tn*5* transposon. The *npt*II ORF was placed under the control of the *E. coli* hybrid *trc* promoter, in the pKK388-1 vector, transformed into *E. coli* DH5α Δ*rec*A (recombinant, deficient strain). Transferred cells were tested for ampicillin, tetracycline, kanamycin, neomycin, geneticin, paromomycin, penicillin, and UV resistance. The neomycin phosphotransferase gene of *E. coli* was cloned successfully and conferred kanamycin, neomycin, geneticin, and paromomycin resistance to recombinant DH5α; this did not inhibit insertion of additional antibiotic resistance against ampicillin and tetracycline, meaning the *trc* promoter can express two different genes carried by two different plasmids harbored in the same cell. This resistance conferral process could be considered as an emulation of horizontal gene transfer occurring in nature and would be a useful tool for understanding mechanisms of evolution of multidrug-resistant strains.

Key words: *Escherichia coli*; Neomycin phosphotransferase gene (*npt*II); Homologous recombination gene (*rec*A); Aminoglycoside resistance

INTRODUCTION

The neomycin phosphotransferase gene (*npt*II) was initially isolated from the transposon Tn*5* of the bacterium *Escherichia coli* K12. Transposon Tn*5* is a movable DNA element of about 5.3 kbp that carries resistance to the aminoglycoside antibiotics (Berg et al., 1975) and that also seems to encode proteins that participate in the transposition reaction (Rothstein et al., 1981). Furthermore, the presence of transposon Tn*5* was studied in 730 Enterobacteriaceae strains from clinical and sewage origin (Blázquez et al., 2006).

The *npt*II gene encodes neomycin phosphotransferase II (NPTII; EC 2.7.1.95), also called aminoglycoside 3'-phosphotransferase II (APH(3')II), which inactivates by phosphorylation a range of aminoglycoside antibiotics such as kanamycin, neomycin, geneticin, and paromomycin (Berg et al., 1975; Auerswald et al., 1981; Beck et al., 1982; Genilloud et al., 1988). The *neo* gene of the Tn*5* appears to be an excellent selection marker for vectors in prokaryotic as well as in eukaryotic systems (Herrmann et al., 1978; Rao and Rogers, 1979; Jimenez and Davies, 1980; Colbere-Garapin et al., 1981; Southern and Berg, 1982). Besides its use as a selectable marker, the gene is also very attractive for use as a generally applicable indicator gene to examine gene expression and gene regulation (Reiss et al., 1984). For such studies, it would be of advantage if the NPTII could be fused onto a gene product of interest without losing enzymatic activity.

The aim of the present study was to clone the *npt*II gene of the *E. coli* K12 strain, by placing its open reading frame (ORF) under the control of a powerful promoter, in an expressive plasmid in *E. coli*.

E. coli DH5α (Δ*rec*A) strains were selected to be transferred by plasmid harboring the *npt*II gene because Δ*rec*A strains have the advantage of stably maintaining introduced plasmids.

This study would permit the evaluation of the ability of *trc* promoter to express two different genes carried by two different plasmids harbored by the same cell. In addition, this study would help to evaluate the effect of the presence of functional *rec*A in a bacterial cell on its efficiency in receiving and expressing another gene carried on another plasmid, such as the *npt*II gene.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Tables 1 and 2 show the bacterial strains and vectors used in this study. *E. coli* was grown aerobically at 37°C in Luria-Bertani (LB) medium (Difco). Growth was monitored by $OD_{600\ nm}$. Ampicillin (100 µg/mL), kanamycin (25 µg/mL), tetracycline (10 µg/mL), neomycin (25 µg/mL), geneticin (10 µg/mL), paromomycin (10 µg/mL), and penicillin (25 µg/mL) were added to the medium when needed. All chemicals were purchased from Sigma.

DNA manipulations

Standard molecular biology procedures were used according to Sambrook et al.

(1989). Enzymes were obtained from Boehringer Mannheim. Plasmid DNA was prepared with the Wizard kit from Promega. DNA fragments were isolated from agarose gels with the Jetsorb kit (Genomed). The colonies kept for further characterization were purified once by single-colony isolation in selective solid medium. Stationary-phase cultures, grown from a single colony, were added to 1 mL liquid medium, mixed with 1 mL 80% glycerol and stored at -20°C.

Table 1. Bacterial strains used in this study.

Strain	Principal characteristics	Source or reference
Escherichia coli K12	F^+, lambda$^+$	(Migula, 1895; Castellani and Chalmers, 1919), ATCC 10798
Escherichia coli DH5α	F^-, Δ (*lac-argF*)U169, *recA1*, *endA1*, *hsdR17*($r_k^- m_k^+$), *supE44*, *gyrA*1, *relA1*, *deoR*, *thi-1* (φ 80*dlacZ* Δ M15)	(Woodcock et al., 1989), (Gibco-BRL)

ATCC = American Type Culture Collection, Rockville, MD, USA.

Table 2. Vectors used in this study.

Vector	Principal characteristics	Source or reference
pGEM-T (Easy)	*E. coli* cloning vectors for the PCR products, origin of replication ColE1, Amp^r *bla lacI orif1* (pGEM-T and pGEM-T Easy, differing by the restriction sites present around the insertion site)	Promega
pKK388-1	*E. coli* expression vector with *trc* promoter, Apr	(Brosius, 1988), Clontech, Inc.
pNPT	pGEM-T Easy vector overhanging *npt*II ORF	This study
pNPTR	Plasmid derived from pKK388-1, containing *npt*II ORF under p*trc* control	This study
pSH2	Plasmid derived from pKK388-1, *rec*A ORF in the form of fragment *Nco*I/*Sma*I under p*trc* control	(El Shafey et al. 2009)

Promega Corporation Clontech Laboratories Inc.

Analysis of the nucleotide sequences

The CLC Sequence Viewer Software, GeneJockey program, DNA Strider, Blast (NCBI), and CLUSTAL W allowed the analysis of the nucleotide and amino acid sequences.

Polymerase chain reaction (PCR) amplification

The oligonucleotides described in Table 3 were used as primers. PCR was carried out with 2.5 U thermostable DNA polymerase (AmpliTaqGold from Perkin-Elmer) in a reaction mixture containing an *E. coli* plasmid DNA, 0.2 mM deoxynucleotide triphosphates (Promega), 0.5 µM of both primers, 2 mM $MgCl_2$ and 1X AmpliTaq buffer in a final volume of 50 µL. For the amplification reaction, after 10 min at 94°C, 25 identical cycles (1 min of denaturation at 94°C, 1 min of hybridization at 50°C, 1 min of elongation at 72°C) were followed by a final elongation step of 5 min at 72°C. The amplified DNA fragment of the expected size was cloned into the pGEM-T vector (Promega).

Ultraviolet (UV) resistance assays

To measure UV resistance in *E. coli*, a fresh culture was initiated from an overnight culture and incubated for 3 h (logarithmic phase of growth). Next, 2.5 mL culture was placed on glass Petri dishes of 5.5 cm in diameter. Uncovered plates were exposed to 0.004, 0.008, 0.012, 0.016, 0.020, 0.024, 0.028, 0.032 J/cm^2 UV light. Five replicates were carried out for UV exposure experiments. Precautions against exposure of Petri dishes to light after irradiation were taken to avoid a photoreactivation phenomenon. Relative survival at each dose was calculated by comparing the number of colony-forming units (CFU) in the irradiated sample to the number of CFU in the non-irradiated sample.

RESULTS AND DISCUSSION

Analysis of the *npt*II gene sequence

The *npt*II sequence of Tn*5* transposon of *E. coli* K12 ATCC 10798 (Figure 1) was analyzed *in silico* and found to have a molecular mass of 257.378 kDa. Restriction sites were searched using the RestrictionMapper software. The sequence statistics of the *npt*II gene were calculated with the help of the CLC Sequence Viewer Software (Table 3).

Table 3. Base distribution.

Base	Count	Frequency
Adenine (A)	144	0.181
Cytosine (C)	225	0.283
Guanine (G)	249	0.313
Thymine (T)	177	0.223
C+G	474	0.596
A+T	321	0.404

Designing of primers

Two degenerate primers were designed based on the *npt*II sequence (Beck et al., 1982) of Tn*5* transposon of *E. coli* (Figure 1). The Primer3 web designer tool (University of Massachusetts Medical School, USA) was used for designing the two primers. Two primers, each with a length of 21 bases, were created with a calculated G/C percentage of 38.10 and 47.62 for the left primer (*npt*IIpr1) and right primer (*npt*IIpr2), respectively. Melting temperature of the primer was calculated and found to be 58.56° and 59.47°C for *npt*IIpr1 and *npt*IIpr2, respectively. The self-complementarity score of the primers (taken as a measure of its tendency to anneal to itself or form secondary structure) was found to be 4.00 and 3.00 for *npt*IIpr1 and *npt*IIpr2, respectively. While the 3' self-complementarity of the primers (taken as a measure of its tendency to form a primer-dimer with itself) was found to be 2.00 for both primers. Primer sequences are shown in Table 4.

Table 4. Oligonucleotides used in this study.

Name	Sequence (5'-3')	Restriction site present
*npt*IIpr1: tgattgaacaagatggattgc	(21 b)	*Bcc*I, *Ags*I
*npt*IIpr2: gaactcgtcaagaaggcgata	(21 b)	*Psh*AI

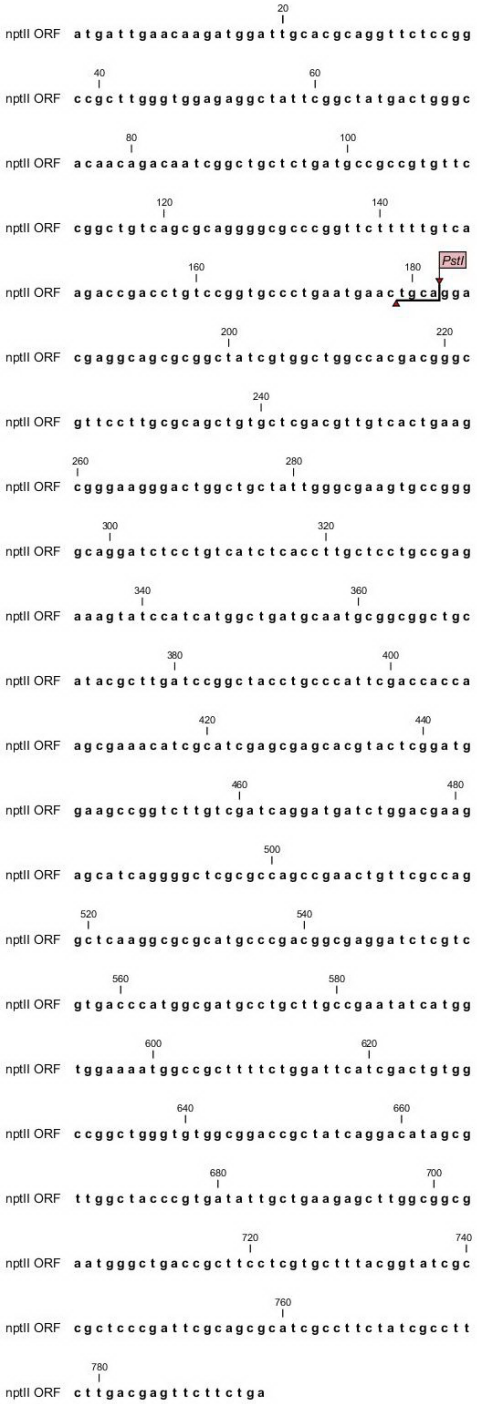

Figure 1. *npt*II sequence of Tn*5* transposon of *Escherichia coli* K12 (ATCC 10798).

Cloning of the *npt*II gene into the pGEM®-T Easy vector

The extracted plasmid DNA of *E. coli*, together with the degenerate primers, were used in PCR experiments, for amplifying the *npt*II gene.

A fragment of 795 bp corresponding to the *npt*II gene was amplified. The pGEM®-T Easy vector was selected to be used in the first step because it is convenient for cloning PCR products, as it is an open vector with a single 3' terminal thymidine (T) that overhangs at the insertion site to both ends. The presence of these single 3'-T overhangs at the insertion site, greatly improves the efficiency of ligation of a PCR product into the plasmids by providing a compatible overhang for PCR products generated by certain thermostable polymerases (Mezei and Storts, 1994; Robles and Doers, 1994). It often adds a single deoxyadenosine in a template-independent fashion, to the 3'-ends of the amplified fragments (Clark, 1988; Newton and Graham, 1994).

The PCR fragment extracted from the gel was cloned in the pGEM®-T Easy vector, resulting in a pNPT plasmid (Figure 2).

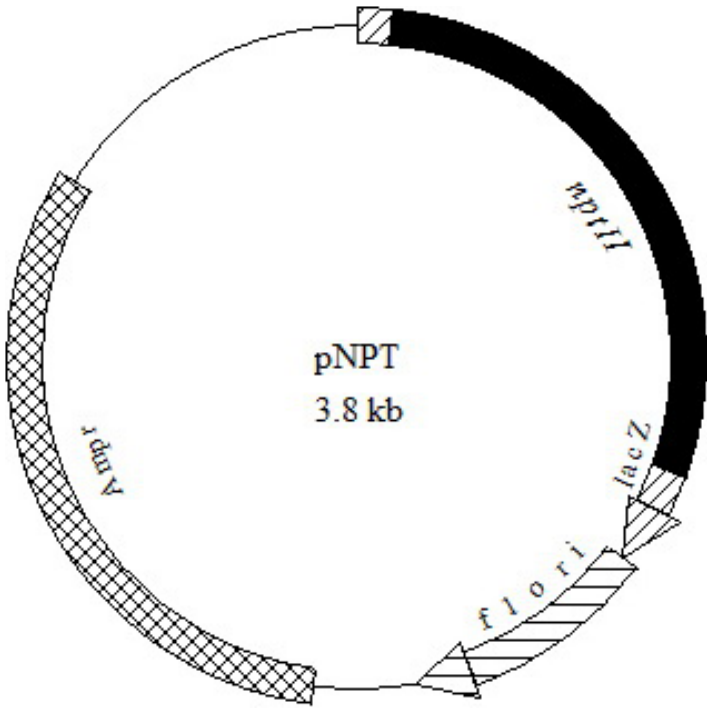

Figure 2. pNPT.

Many studies reported successful cloning of many bacterial aminoglycoside antibiotic genes, such as in *E. coli* (Clerget et al., 1982), *Campylobacter jejuni* (Tenover and Elvrum, 1988), *Bacillus circulans* (Sarwar and Akhtar, 1990), *Pseudomonas aeruginosa* (Schwocho et al., 1995), *Citrobacter freundii* (Wu et al., 1997), *Streptomyces tenebrurius* (Skeggs et al., 1987), and *Campylobacter coli* (Wang and Taylor, 1990).

Cloning of the *npt*II gene into the pKK388-1 plasmid

The second step was to clone the *npt*II gene extracted from the pNPT plasmid into pKK388-1. pKK388-1 is an *E. coli* expression vector with *E. coli* hybrid *trc* promoter and Ap^r (Brosius, 1988). The *E. coli* hybrid *trc* promoter was previously shown to promote efficient expression of genes in both *E. coli* (Brosius, 1988; El Shafey et al., 2009), and *Corynebacterium glutamicum* (Delaunay et al., 1999).

For this purpose, the pNPT plasmid was digested with *Eco*RI. This site was selected because it is absent in the PCR fragment and is represented in the pGEMT-Easy vector surrounding the insert; yet, at the same time, it is unique in the pKK388-1 plasmid and is found inside its polylinker.

The digested pNPT plasmid was then electrophoresed to separate the digested fragment away from the rest of the plasmid. The fragment of the expected size was purified and extracted from the gel. On the other hand, pKK388-1 was open by *Eco*RI digestion. The extracted PCR fragment was then cloned in the open pKK388-1 plasmid, resulting in pNPTR (Figure 3).

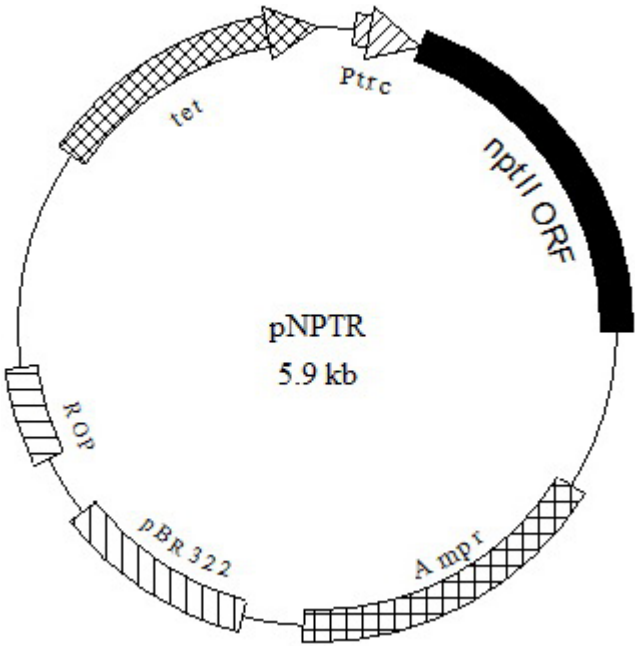

Figure 3. pNPTR.

Expression of recombinant aminoglycoside antibiotic genes in *E. coli* cells has been reported by many authors (e.g., Tenover and Elvrum, 1988; Sarwar and Akhtar, 1990; Wang and Taylor, 1990).

Transformation of pSH2 into DH5α

Plasmid pNPTR was dialyzed and transformed in the *rec*A^- and *rec*A^+ DH5α strains

after its treatment to render it competent for receiving genetic materials, in an attempt to express *npt*II gene in the two stains.

*E. coli rec*A$^-$ DH5α harboring no plasmids were sensitive to ampicillin (Ap^r) and UV, while *rec*A$^+$ cells harboring pSH2 (Figure 4) were resistant to ampicillin, tetracycline and UV up to a dose 0.24 J/cm^2. Cells grown on LB only (non-transformed DH5α), LB + ampicillin plates (*rec*A$^+$, *npt*II$^-$ DH5α::pSH2), and LB + neomycin (*rec*A$^+$, *npt*II$^+$ DH5α::p) were picked up.

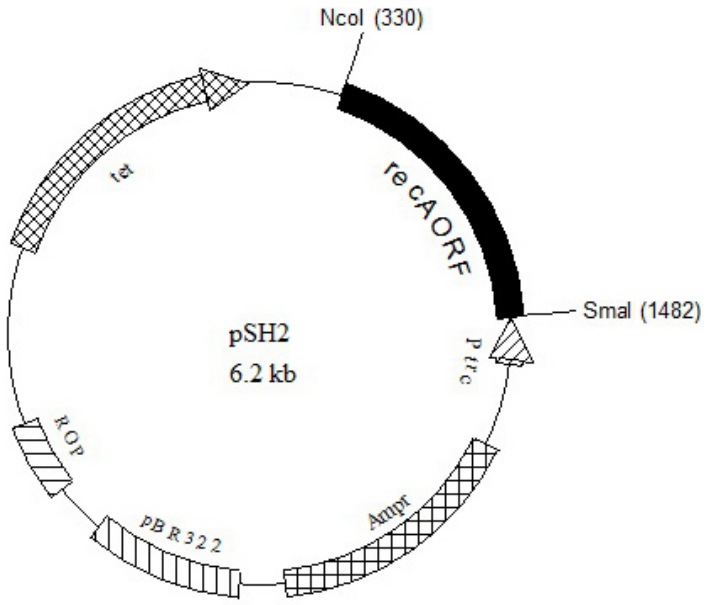

Figure 4. pSH2.

Transformed and non-transformed cells were tested for their sensitivity to the following antibiotics: ampicillin, kanamycin, chloramphenicol, tetracycline, neomycin, geneticin, paromomycin, and penicillin; in addition to their sensitivity to UV doses up to 0.24 J/cm^2.

The results (Table 5) showed that *npt*II-transformed cells exhibited resistance to ampicillin, tetracycline, kanamycin, neomycin, geneticin, and paromomycin, while *E. coli* DH5α exhibited resistance to ampicillin and tetracycline only, and DH5α non-transformed cells did not exhibit resistance to any antibiotic tested.

Table 5. Sensitivity of transformed and non-transformed *Escherichia coli* DH5α cells to different antibiotics.

Strain	None	Amp	Tet	Kan	Neo	Par	Gen	Pen
E. coli DH5α (*recA*$^-$, *npt*II$^-$)	+	-	-	-	-	-	-	-
E. coli DH5α (*recA*$^+$, *npt*II$^-$)	+	+	+	-	-	-	-	-
E. coli DH5α (*recA*$^+$, *npt*II$^+$)	+	+	+	+	+	+	+	-

None = no antibiotic added; Amp = ampicillin; Tet = tetracycline; Kan = kanamycin; Neo = neomycin; Par = paromomycin; Gen = geneticin; Pen = penicillin.

Functionality of the *rec*A gene was proved by relatively high resistance of DH5α transformed by (pSH2::*rec*A) and (pNPTR::*npt*II) equally to UV up to a dose 0.24 J/cm^2,

while non-transformed DH5α (Δ*rec*A) was sensitive to the tried range of UV (Figure 5).

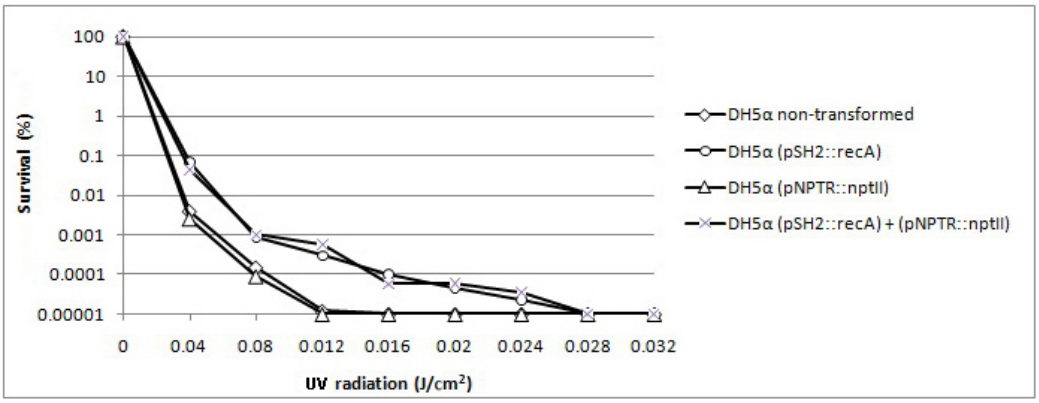

Figure 5. Functionality of *rec*A gene expressed as relative tolerance of transformed and non-transformed *Escherichia coli* cells to UV radiation.

We concluded that the neomycin phosphotransferase gene of *E. coli* was cloned successfully and proved to confer kanamycin, neomycin, geneticin, and paromomycin resistance to recombination-positive DH5α, which in turn did not inhibit insertion of a new antibiotic resistance to the same cell already having ampicillin and tetracycline resistance genes, indicating the ability of the *trc* promoter to express two different genes carried by two different plasmids in the same cell.

It would also be interesting to follow up on this study with researchs concerned with the expression of more than two plasmids harboring different genes belonging to different families of antibiotic resistance genes cloned under the control of the *trc* promoter. The resulting recombinant strains would be of special interest for the emulation of the evolutionary mechanisms of resistance based on horizontal transfer of resistance genes. Such studies would also be interesting for the evaluation of new antibiotic generations destined for multidrug resistant strains that have evolved in last few years (Sandel et al., 2002; Lim et al., 2009; Jan et al., 2009; Johnson, 2010) as a result of the extensive use of different antibiotic drugs.

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