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Eric S. Nazareno
University of Minnesota - Twin Cities

Bimala Acharya
Tennessee State University

C. Korsi Dumenyo
Tennessee State University

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DR. C. KORSI DUMENYO (Orcid ID : 0000-0002-7846-9090)

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A mini-Tn5-derived transposon with reportable and selectable markers enables rapid generation and screening of insertional mutants in Gram-negative bacteria

Eric S. Nazareno[1], Bimala Acharya[2], and C. Korsi Dumenyo[2]*

[1]Department of Plant Pathology, University of Minnesota, St. Paul, MN, 55108, USA

[2]Department of Agricultural and Environmental Sciences, Tennessee State University, Campus Box 9543, Nashville, TN, 37209, USA

*Corresponding author: cdumenyo@tnstate.edu

Running headline: Multi-function Tn5 transposon

Significance and impact of the study

Transposon mutagenesis remains a powerful and inexpensive tool in bacterial functional genomics. We developed a transposon with multiple combined features that allow selection of inducible or expressed genes, real-time quantification of gene expression levels using promoter-less GFP, and rapid cloning and identification of the inactivated gene. Our Tn5-derived transposon worked in mutagenizing bacteria in the Enterobacteriaceae, Pectobacteriaceae and Pseudomonaceae families, indicating a broad range application. Using the transposon, we generated a high quality and saturated mutant library of *P. versatile*. Our high efficiency transposon is customizable and has different options for delivery and antibiotic selection, which make it a promising tool for molecular studies.

Abstract

We re-engineered a classic tool for mutagenesis and gene expression studies in Gram-negative bacteria. Our modified Tn5-based transposon contains multiple features that allow rapid selection for mutants, direct quantification of gene expression, and straightforward cloning of the inactivated gene. The promoter-less *gfp-km* cassette provides selection and reporter assay depending on the activity of the promoter upstream of the transposon insertion site. The *cat* gene facilitates positive antibiotic selection for mutants, while the narrow R6Kγ replication origin forces transposition in recipient strains lacking the *pir* gene and enables cloning the transposon flanked with the disrupted gene from the chromosome. The suicide vector pCKD100, a plasmid that could be delivered into recipient cells through bi-parental mating or electroporation, harbors the modified transposon. We used the transposon to mutagenize *Pectobacterium versatile* KD100, *Pseudumonas coronafaciens* PC27R, and *Escherichia coli* 35150N. The fluorescence intensities of mutants expressing high GFP could be quantified and detected qualitatively. Transformation efficiency from conjugation ranged from 1600 to 1900 CFU ml^{-1}. We sequenced the upstream flanking regions, identified the putative truncated genes, and demonstrated the restoration of the GFP phenotype through marker exchange. The mini-Tn5 transposon was also utilized to construct mutant library of *P. versatile* for forward genetic screens.

Keywords: Tn5 transposon; transposon mutagenesis; multi-function; gene expression; promoterless GFP; *Pectobacterium versatile*; *Pseudomonas coronafaciens*; *Escherichia coli*

Introduction

Microbial genetics has benefited from the discovery of transposons and their development into traditional experimental tools for understanding gene regulation and other cellular processes in prokaryotes (Weinstock, 2001; Hayes, 2003; Kulasekara, 2014). Among the three major classes of genetic elements in bacteria, which include insertion sequences, transposons (Tn3, Tn5, Tn7, and Tn10), and transposable phages, Tn5 transposon derivatives have been primarily adapted for laboratory use due to the randomness of their insertion and ability to function in different hosts (Reznikoff, 2008; Barquist, Boinett & Cain, 2013; Srivastava, 2013). The wild type Tn5 is a 5.8 kb composite transposon containing three antibiotic genes flanked by two nearly identical insertion sequences, IS50L and IS50R, which are placed in inverted orientations and are defined by 19 bp outside end (OE) and inside end (IE) sequences (Reznikoff, 2008; Srivastava, 2013). Modifications of the Tn5 system led to the generation of derivatives known as mini-Tn5 transposons, which are usually smaller than 5 kb and may contain selectable antibiotic markers and reporter genes such as *lacZ*, *gfp*, *phoA*, *luxAB*, and *xylE* to monitor gene expression (de Lorenzo *et al.*, 1990; de Jong & Geiselmann, 2015).

Green fluorescent protein (GFP) and it variants have been widely used as autofluorescent genetic reporters in prokaryotic and eukaryotic systems since its cloning in 1992, although first discovered from *Aequorea victoria* in 1960 (Carroll & James, 2009). The main advantages of GFP over other reporter systems are that it is generally non-toxic to cells and its expression can be observed and quantified in real-time without the need for substrates or lengthy assay protocols. (Soboleski, Oaks & Halford, 2005). Although several variants of GFP have been developed and utilized for gene expression studies, GFP-Uv, a less-toxic variant that fluoresces in a broad pH range (5.5-12.0), has been optimized for increased fluorescence intensity in bacteria (Tang, Lu & Pan, 1999; Feilmeier *et al.*, 2000; Hadjantonakis & Nagy, 2001). Promoterless GFP transposon systems have been used for forward genetic screens to identify genes that control important phenotypes in prokaryotes (Dunn, Klimowicz & Handelsman, 2003; Lyell *et al.*, 2008; Martínez-García *et al.*, 2011). However, these systems do not possess all the convenient features for positive antibiotic selection, quantification of gene expression, and cloning of the disrupted gene. Gene expression studies in bacteria can also be conducted using microarrays and more recently, RNA-seq (Schoolnik, 2002; Poulsen & Vinther, 2018). Though microarrays have become obsolete, both methods are considerably more expensive than

transposon mutagenesis. In this paper, we present an efficient and cost-effective tool for identifying causative genes in Gram-negative bacteria using a promoter trap system.

Results and Discussion

We constructed a new suicide vector containing a modified transposon by cloning the *gfp-km* cassette from pTGN and using pTnMod-RCm as the backbone. To ensure that the *gfp-km* cassette will be running downstream of a truncated gene when the transposon inserts in the forward orientation (*gfp-km* in the 5' end), we employed a two-step cloning procedure that involved producing fragments that contain both a sticky and a blunt end and subsequently ligating the compatible ends of the two fragments together. The size of the new plasmid is 6.2 kb and the multiple features of its 3.2 kb Tn5 transposon include a narrow-range R6Kγ origin of replication, chloramphenicol (*cat*) resistance driven by a constitutive promoter, which allows positive antibiotic selection, and a promoterless *gfp-km* cassette that can be used for promoter tagging for expressed genes under desired conditions (**Figure 1**). The R6Kγ origin restricts proliferation of the plasmid in recipient cells lacking the *pir* structural gene, thereby forcing the transposon to integrate into the chromosome. The transposon also provides a replication origin for cloning the flanking sequences as a plasposon for determining the truncated gene and transfer of the mutation into other strains with isogenic backgrounds (Dennis & Zylstra, 1998). If obtaining mutants is the priority regardless of GFP expression, selecting on chloramphenicol alone with the appropriate antibiotic for the recipient would enable generating a mutant library. Finally, to maximize the utility of the transposon, the reporter activity of the *gfp-km* operon can be used to quantify gene expression levels of the upstream gene by selecting on kanamycin (*nptII*). A translational signal, *atpE*, is also included in the GFP and NptII open reading frames, allowing transcriptional fusions without the need for in-frame insertions (Tang *et al.*, 1999). The transposon provides for the generation of stable mutations as the transposase gene is outside of the inverted repeats. In addition to these features, the availability of multiple cloning sites immediately within the inverted repeats makes the vector amenable to further modifications. Moreover, pCKD100 is maintained in S17-1 λpir, which does not need a helper during conjugation.

Results from the initial transposon mutagenesis experiment via bi-parental mating showed that the suicide vector has a transformation and transposition efficiency of 1800 CFU ml-

$^{-1}$ in *P. versatile* KD100, 1600 CFU ml^{-1} in *P. coronafaciens* PC27R, and 1900 CFU ml^{-1} in *E. coli* 35150-N per 2x2 cm nylon membrane mating discs. Selection for chloramphenicol resistance together with either nalidixic acid or rifampicin enabled construction of a small mutant library for each species. Given that the transposon can insert in the chromosome in one of two orientations during an insertion event (Atkinson *et al.*, 2018; Goodall *et al.*, 2018), the probability that the promoterless *gfp-km* operon can be driven by an upstream promoter is approximately 50%. Thus, about half of the mutants in the library are theoretically expected to have an insert where *gfp-km* is downstream of a promoter. However, due to the small number of exconjugants tested, we were not able to confirm this, but phenotypically, we observed different degrees of fluorescence when mutants were viewed under a handheld UV lamp (365 nm). Since we randomly selected mutants that are fluorescent, majority of them exhibit some levels of GFP expression in our test. The phenotypes of some GFP mutants and the wild type parents are shown in **Figure 2**. We also tested electroporation as a method of delivery of the vector into recipient cells but observed a much lower transformation efficiency (data not shown) compared to conjugation, an observation that had also been indicated previously (Dennis & Sokol, 1995).

For the fluorescence assay, 30 GFP-expressing insertional mutants per library of *P. versatile*, *P. coronafaciens*, and *E. coli* (90 mutants in total) were selected for GFP quantification to assess the utility of the transposon for promoter tagging. The relative fluorescence intensities of the GFP mutants ranged from 0 to 4000 relative fluorescence units (RFU) (**Figure 3**), which conform with the data from the first GFP-Uv assay using *gfp-km* (Tang *et al.*, 1999). Among the three mutant libraries, *P. coronafaciens* PC27R-GFP exhibited the widest spread in RFU, followed by *P. versatile* KD100-GFP and then by *E. coli* EC35150N-GFP. The PC27R-GFP pool also had the mutants with the highest RFU, with two reaching more than an average of 2000 units (**Figure 4**). Conversely, fluorescence intensities in the EC35150N-GFP library were the lowest and only reached a maximum of 390 RFU, while the peak RFU for most of the mutants in all libraries is about 250. The varying GFP levels can be an evidence of random insertion of the transposon, though the site of insertion within a gene could also affect GFP expression. Nevertheless, Dennis and Zylstra (1998) tested the randomness of the pTnMod system, from which our transposon was derived, and they demonstrated random transposition. The GFP-expressing mutants were also resistant to kanamycin at 50 µg ml^{-1}.suggesting that the *gfp-nptII* cassette could also be an effective selection marker. This experiment demonstrates the rapid

identification of insertional mutants and direct quantification of gene expression levels using the reporter activity of GFP and kanamycin as the selectable marker.

We could not rule out the possibility of cryptic promoter activity in the *gfp* gene that could drive the expression of the downstream *nptII* gene. The possible existence of such promoter activity is important for two reasons. First, Tang et al., (1999) previously reported that *Agrobacterium* mutants created with the predecessor transposon, pTGN, that had minimal GFP activity still had high resistance to kanamycin. Thus, suggesting that the high kanamycin resistance could result from the activity of a supplementary promoter located withing the *gfp* gene. Secondly, we also observed leaky kanamycin resistance but no fluorescence activity when the *E. coli* S17-1λPir carried pCKD100. However, in three strains of *Erwinia tracheiphila* which also replicates the plasmid, there was neither GFP activity nor kanamycin resistance (data not shown). These observations suggest that even if there is cryptic promoter activity within the *gfp* gene, it will be species dependent.

After partial digest of the mutant genomic DNA, we cloned the plasposon and demonstrated that the transposon insertion could be exchanged into a wild type isogenic genome by electroporating the cloned plasposon into the wild type strains and observing GFP expression. In addition, since all cultures were assayed in either LB or KB with appropriate antibiotics but without the presence of any inducer, we expected that most essential genes would be activated. Sequencing of re-ligated fragments from the partial digest of the genomic DNA from three representative mutants revealed that some of the putative highly expressed genes based on BLAST alignments consist of housekeeping genes such as the 23S ribosomal RNA (**Table 1**). There are up to five copies of the 23S ribosomal RNA in *P. coronafaciens* genome (NCBI accession: PRJNA591322), thus, deactivation of a single copy would not be deleterious. Naorem *et al.* (2018) also found 23S rRNA as one of the insertion sites of their mini-Tn5 transposons in *P. aeruginosa* PAO1. For the *E. coli* 35150N mutant, BLAST alignments showed multiple hits in several *E. coli* reference genomes, but the top hit is a DUF2931 family protein gene, which was suggested to be part of an outer membrane lipoprotein that inhibits a T6SS toxin effector required for interbacterial competition (Flaugnatti *et al.*, 2016). Lastly, a DNA-binding protein, amino acid permease and AEC transporter (auxin efflux transporter similar to ABC transporters), the latter two being both involved in amino acid transport (Quintero *et al.*, 2001; Zazímalová *et*

177 *al.*, 2010), are the putative genes for the KD100 mutant based on BLAST alignments using *P.*
178 *carotovorum* and *P. versatile* reference genomes.
179 We also used the Tn5 transposon to construct a large and high-quality library of 36,000
180 KD100 mutants by selecting on both chloramphenicol and kanamycin to only include forward
181 insertions with GFP fusions. Preliminary screening of the library revealed interesting phenotypes
182 such as mutants that overproduce plant cell wall-degrading enzymes (PCWDEs) including
183 pectate lyase, polygalacturonase, cellulase, and protease, allowing them to be more pathogenic to
184 multiple hosts (unpublished data). Uncovering the genes that are responsible for these
185 phenotypes would help understand the molecular basis of soft rot pathogenesis, which further
186 demonstrates the utility of the transposon as a useful tool for genetic studies in prokaryotes. In
187 conclusion, we have constructed a mini-Tn5-derived transposon with multiple utilities that can
188 be used for isolation of both reportable and selectable mutants in a wide range of Gram-negative
189 bacteria.
190
191 **Materials and Methods**
192 **Bacterial strains and plasmids**
193 *P. versatile* KD100 and *E. coli* 35150N were maintained in Luria Broth (LB)
194 supplemented with nalidixic acid (50 µg ml^{-1}) at 28°C and 37°C, respectively; *P. coronafaciens*
195 PC27R was maintained in King's Broth (KB) with rifampicin (50 µg ml^{-1}) at 28°C. Overnight
196 cultures were used in all the experiments.
197 The 4.4 kb plasmid pTnMod-RCm (Dennis & Zylstra, 1998) was digested with *Spe*I then
198 treated with Klenow fragment of *E. coli* DNA polymerase to fill in the 5' overhang and produce
199 blunt ends. The blunt ended fragment was digested with *Not*I and purified from agarose gel using
200 GeneClean Kit (MP Biomedicals, CA, USA). A 1.8 kb fragment containing the *gfp-km* cassette
201 was cleaved from pTGN (Tang *et al.*, 1999) using *Sma*I and *Not*I and purified as described
202 above. The *Not*I sticky ends and *Sma*I-*Spe*I/Klenow blunt ends were ligated to form a new 6.2 kb
203 plasmid, named pCKD100 (**Figure 1**). The plasmid was electroporated to a suitable donor, S17-1
204 λ*pir*, which is a *pir*-expressing strain that supports the replication of the R6Kγ origin, and then
205 mutants were selected using the antibiotics described above.
206 **Transposon mutagenesis**

Using bi-parental mating, pCKD100 was delivered from the S17-1 λpir donor to the recipients, *P. versatile* KD100, *P. coronafaciens* PC27R, and *E. coli* 35150N. Overnight cultures were spun down and resuspended in LB. Bacterial concentrations were standardized at A_{600}= 1.0 using Synergy H1 Hybrid Reader (BioTek Instruments, VT, USA). Equal volumes of S17-1 λpir/pCKD100 and the recipients were mixed and an aliquot of 200 µl was pipetted to four 2x2 cm magnaprobe nylon membranes (GE Water and Process Technologies, PA, USA) on LB agar plates. The plates were incubated overnight, and the mating mixture was dislodged from the membranes by transferring them into 2 ml microcentrifuge tubes and homogenizing with 30% glycerol + LB broth. Serial dilutions were made and mutants were obtained by selecting on chloramphenicol (30 µg ml^{-1}) agar plates with nalidixic acid (25 µg ml^{-1}) for *P. versatile* KD100 and *E. coli* 35150-N or chloramphenicol and rifampicin (50 µg ml^{-1}) for *P. coronafaciens* PC27R exconjugants to eliminate donor cells.

GFP detection and quantification

GFP-expressing mutants were detected using a handheld UV lamp (Analytik Jena, Upland, CA, USA). From the mutant pool, 30 colonies per recipient strain exhibiting varying degrees of fluorescence were randomly selected. By replica plating using a pin stamp, 95 colonies including controls were inoculated to a 96 black-walled microplate (BD Biosciences, NJ, USA) with 200 µl LB per well and shaken overnight at 28°C. Fluorescence was quantified using the Synergy H1 Hybrid Reader with excitation at 423 nm and emission at 509 nm in four replicates. Relative fluorescence intensities were computed based on the following formula by Tang *et al.* (1999), with an additional correction for the absorbance of the culture medium:

$$I_R = I_{abs}/OD_{600} - I_C/OD_C - I_M$$

where I_c is the I_{abs} of the wild type; OD_c is the OD_{600} of the wild type; I_M is the I_{abs} of the medium

Partial digest and marker exchange

Five mutants per strain were selected for partial digest. Genomic DNA was extracted using the Promega Wizard genomic DNA kit (Promega, Madison, WI, USA). About 5000 ng DNA per mutant was partially digested with *Sau*3A for 15 min at 37°C. The reaction was stopped by adding 1 µl of 500mM EDTA and incubating for 20 min at 70°C. The digest was run in an agarose gel and fragments greater than 5 kb were purified. Purified fragments were self-

ligated and electroporated to S17-1 λpir. Plasposon DNA was extracted and then electroporated to the wild type strains to observe if GFP expression would be restored.

Sequencing and gene identification

The primer pTGN_Gfp_Seq (5' CAG TTT GTT TCA GTT AAA AC 3') designed from the AtpE locus was used to sequence from the *gfp* end of the transposon. Partially digested DNA from eight mutants from the three mutant libraries were randomly selected for Sanger sequencing of the upstream region (~600 bp). The sequences obtained were aligned against the available reference genomes of *E. coli*, *P. coronafaciens*, *P. carotovorum*, and *P. versatile* in NCBI using megablast and BLASTN with default parameters, and putative genes were deduced from the BLAST results.

Construction of a KD100 mutant library with 36,000 mutants

Bi-parental mating between S17-1 λpir and *P. versatile* KD100 was performed as described above. An aliquot of 100 µL from the overnight incubated mating mixture was spread on nutrient gelatin (NG) plates with recommended doses of Nal, Cm and Km, and incubated at 28°C for 3 days. After the bacterial colonies appeared on selection plates, they were washed with 1 mL of LB broth with the selection antibiotics and transferred into a culture flask. The culture of washed transconjugants from all the plates was incubated overnight with shaking at 200 rpm, then centrifuged at 3836g at 28°C and the supernatant was discarded. Pellet was resuspended and homogenized in 30% glycerol + LB liquid medium, then 100 µL of resuspended pellets was aliquoted in 0.5 mL microcentrifuge tubes and stored in -80°C freezer.

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Availability of the plasmid construct

The sequence of pCKD100 has been deposited at Genbank under accession number MW039600. The construct is also available to the research community and can be obtained from addgene.

Conflict of interest

The authors declare no conflict of interest.

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Table 1. Putative highly expressed genes identified from three representative pCKD100 insertional mutants.

Flanking sequence source	Reference genome	BLAST alignment	Coverage	E-value	Identity
PC-3 (*P. syringae* pv. *coronafaciens* PC27R mutant)	*P. coronafaciens* pv. *coronafaciens* strain B19001	23S ribosomal RNA	82%	9e-57	87%
	P. coronafaciens pv. *oryzae* str. 1_6	23S ribosomal RNA	82%	9e-57	87%
EC-11 (*E. coli* 35150-N mutant)	*E. coli* strain 214-4	DUF2931 family protein	50%	2e-54	82.5%
	E. coli strain FDAARGOS_403	glucans biosynthesis glucosyltransferase MdoH	50%	3e-52	82%
	E. coli strain FDAARGOS_401	LysR family transcriptional regulator	50%	3e-52	82%

	E. coli O104:H4 strain FDAARGOS_348	GNAT family N-acetyltransferase	50%	3e-52	82%
	E. coli O104:H4 strain FDAARGOS_349	two-component-system connector protein YmgA	50%	3e-52	82%
	E. coli strain FORC_029	chromosomal replication initiator protein DnaA	50%	3e-52	82%
KD-4 (*P. versatile* KD100 mutant)	*Pectobacterium versatile* strain 14A plasmid pPC14A1	DNA-binding protein	60%	6e-65	80%
	P. carotovorum subsp. *carotovorum* PC1	amino acid permease	6%	0.36	87%
	P. carotovorum subsp. *carotovorum* strain JR1.1	AEC family transporter	10%	1.2	85%
	P. carotovorum subsp. carotovorum strain BP201601.1	AEC family transporter	12%	1.2	85%

Figure Legends

Figure 1. Plasmid map of pCKD100 showing the multi-feature transposon flanked by the inverted repeats (IR). The presence of CmR allows constitutive expression of chloramphenicol resistance while the promoterless *gfp-km* cassette enables selection for highly expressed genes.

Figure 2. Representative GFP mutants showing variations in fluorescence compared to the wild type strains, observed under a handheld UV lamp and selected on Cm30. A) *Pectobacterium*

versatile KD100 mutants, B) KD100 wild type (WT), C) *Pseudomonas syringae* pv. *coronafaciens* PC27R mutants, D) PC27R WT, E) *Escherichia coli* 35150-N mutants, F) 35150-N WT.

Figure 3. Distribution of relative fluorescence units (RFU) in the *Pectobacterium versatile* KD100, *Pseudomonas syringae* pv. *coronafaciens* PC27R, and *Escherichia coli* 35150-N mutant pools. The RFU for each mutant was calculated by dividing the absorbance with OD_{600} and then subtracting the corresponding value obtained from the wild type.

Figure 4. Relative fluorescence intensities of the 90 mutants on LB with Cm30 and Nal50 (KD100-GFP and EC35150N-GFP), and KB with Cm30 and Rif50 (PC27R-GFP). Fluorescence was measured using Synergy H1 Hybrid Reader with excitation at 423 nm and emission at 509 nm in four replicates.