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An improved Tn7-*lux* reporter for broad host range, chromosomally-integrated promoter fusions in Gram-negative bacteria

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Abstract

An improved vector for chromosomally-integrated promoter-*lux* fusions is described. The modified vector was tested in parallel with the unmodified vector using the well-characterized *E. coli* *araBAD* promoter in the *Pseudomonas aeruginosa* *attTn7* site. The modified mini-Tn7 showed reduced background luminescence, and increased luminescence upon induction, giving >16-fold higher induction ratio.

Keywords

luciferase; *lux*; reporter; Tn7; *araBAD*

The use of reporter fusions is a powerful means for assessment of bacterial gene regulation. There are many vehicles for the cloning of promoters upstream of various reporter genes and subsequent transfer into bacteria. A particularly useful reporter is the bacterial luciferase system or *luxCDABE* genes which allow automated real-time monitoring of gene expression without the need for sample removal or *in-vitro* assays (8). The use of mini-Tn7 elements allows single-copy chromosomal integration into genomes with the highly-conserved, orientation-specific, intergenic and neutral Tn7 attachment site (*attTn7*) (6). The well-described insertion site specificity also affords an ability to avoid problems associated with position effect on reporter activity within a genome (1) and complications arising from multiple (e.g. plasmid-borne) copies of promoters which may titrate cellular regulatory factors. Additionally, the use of antibiotics to maintain plasmid replicons may have unanticipated physiological effects. Shen *et al.* constructed mini-Tn7-*lux* vectors with an improved ribosome binding site upstream of the *Photobacterium luminescens* *luxCDABE* genes, versatile multiple cloning sites, and various selectable markers (10). Choi and Schweizer described an extensive set of mini-Tn7 derivatives that included a mini-Tn7-*lux* construct (pUC18-mini-Tn7T-Gm-*lux*) with the added feature of FLP recombinase-mediated

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removal of antibiotic resistance cassettes (2). That feature minimizes potential artifacts arising from effects on adjacent genes or from the expression of exogenous antibiotic resistance-encoding genes. More recently, Damron *et al.* described a modification to that system (pUC18T-mini-Tn7T-lux-Gm) allowing conjugal transfer (3). Those constructs feature the *luxCDABE* genes of *P. luminescens* with 469 bp of upstream, non-coding DNA between the multiple cloning site and the *lux* cassette. That non-coding DNA was not characterized as to its effects on promoter fusion performance, i.e. attenuation or promoter activity. A scan of that sequence using a bacterial promoter-finding software (BPROM, Softberry, Inc.) found a potential promoter within that region (nt 2338 of GenBank accession #AY962893.1). We attempted cloning of bacterial promoters into pUC18-mini-Tn7T-Gm-lux using an *XcmI* site 12 base pairs upstream of the *luxC* translational start site, and a site within the multiple cloning site, to replace those sequences. Sequencing of a clone derived from one such experiment (pAG2) proved it to be an aberrant ligation product with no insert but with a deletion of sequence between the *XcmI* site (nt 2504) and a site 117 base pairs upstream of the multiple cloning site (nt 1892). pAG2 was then utilized for the construction of a new mini-Tn7 vector, that lacked the upstream non-coding sequence, in two steps (Figure 1.)

First, an *EcoRI* cut site was created 18 nt upstream of the *luxC* translational start site (nt 1888) by site-directed mutagenesis (pUCTn7mutFW and RV primers, QuikChange, Agilent Technologies, Santa Clara, CA) to give pAG3. Next, a multiple cloning site was restored by amplifying a segment of pUC18-mini-Tn7T-Gm-lux, containing the gentamicin-resistance cassette and the multiple cloning site, using PCR. PCR included a forward primer that contained an *MfeI* site and a reverse primer that annealed downstream of the multiple cloning site (pUCTn7lux MCS Mfe L, pUCTn7lux MCS R). The resulting PCR product was digested with *EcoRI* and *MfeI*, purified, and ligated into *EcoRI*-digested pAG3. A clone with the desired insert orientation was retrieved and designated pAG4. pAG4 thus represents a deletion of nt 1048–1114 and nt 2036–2503 of pUC18-mini-Tn7T-Gm-lux, and facilitates cloning of promoters using *EcoRI* since a second *EcoRI* cut site at nt 1048 was eliminated. The *XhoI* site of the original MCS was lost in the process as an expedient; creation of an *EcoRI* site was afforded easily by the newly-juxtaposed sequence near the *luxC* start codon whereas creation of an *XhoI* site would have been at a site further removed or would have involved more complicated mutagenesis.

The effects of the modifications described were tested by cloning the *E. coli* *araBAD* promoter, including *araC*, from plasmid pJN105 ((9) GenBank AF179381.1). The promoter segment used was obtained by amplification using a forward primer with an *SpeI* site fused to nt 1469 (pJN105FW *SpeI*) and a reverse primer annealing within the multiple cloning site of that vector (ParaBAD *EcoR*). The 1,318 bp *SpeI*- and *EcoRI*-digested PCR product was introduced into both pAG4 and pUC18-mini-Tn7T-Gm-lux which had been digested with the same restriction enzymes (partial *EcoRI* digest in the case of pUC18-mini-Tn7T-Gm-lux). The sequence of approximately 400 bp surrounding the promoter junctions was confirmed by PCR amplification (pAG4paraVerL, pAG4ParaVerR, pUC18TluxVerR primers) and sequencing using the same primers (Retrogen, Inc., San Diego, CA). The respective clones represent reporter constructs differing by the presence or absence of the

noncoding DNA upstream of the *lux* cassette, allowing a test of the effects of the non-coding sequence on reporter expression. Each construct was transferred to the *Pseudomonas aeruginosa* MPA01 genome via co-electroporation with pTNS1 and verified by PCR ($P_{\text{glmS-down}}$, P_{Tn7R} , $P_{\text{glmS-up}}$, P_{Tn7L}) as described by Choi and Schweizer (2). Briefly, this involved electroporation (Eppendorf Electroporator 2510, 12.5 kV/cm) of the mini-Tn7-containing plasmid (150ng) as a mixture with a Tn7 transposase-encoding plasmid (pTNS1, 200ng) to afford chromosomal integration at the *attTn7* site. Gentamicin-resistant recipient strains were then subjected to FLP-mediated recombination (2) to remove the *aacC1* gentamicin acetyltransferase gene and the constitutive P1 integron promoter (3). The resulting strains were tested for expression of the *lux* cassette, measured as luminescence (BioTek Synergy H4, Thermo/Nunc cat. no. 165306 96-well white plates, M9 0.9% succinate medium (5), 200 μl /well, 37°C, medium agitation speed) and growth (optical density @ 600nm) with a range of arabinose concentrations (Figure 2).

The results show universally higher expression for the modified reporter in the presence of inducer as compared with the original *lux* fusion, and lower background (luminescence in the absence of arabinose), giving a higher induction ratio. The data indicate that the upstream non-coding DNA has promoter activity, evidenced by the higher background luminescence, as well as some mode of attenuating activity, seen by lower maximal expression upon equivalent inducer exposure. It should be noted that FLP-mediated excision of the antibiotic resistance cassette significantly reduced the background luminescence by both constructs (96% for empty pAG4 and 67% for empty pUC18-mini-Tn7T-Gm-*lux*).

To summarize, we describe an improved vector for single-copy, broad host range promoter fusion construction having the advantage of a removable selective marker and which should more accurately reflect the degree of induction displayed by cloned promoters. The vector has proven to be stable and has been used to construct integrated reporters from several bacterial promoters ((7), our unpublished results). We also show quantitative evaluation of the *E. coli* *araBAD* promoter in *P. aeruginosa* using a range of inducer (arabinose) concentrations. Those data are relevant for another useful mini-Tn7 vehicle constructed for regulated expression of cloned genes from the *araBAD* promoter in Gram-negative bacteria (4).

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Highlights

- The Tn7 based reporter vector pUC18TminiTn7lux was modified by removal of 467 bp of sequence upstream of the *luxCDABE* bacterial luciferase and accessory function genes in order to remove potential promoter or attenuator activity
- An *EcoRI* site within the multiple cloning site (MCS) of pUC18TminiTn7lux was also rendered more useful by eliminating an additional *EcoRI* site external to the MCS in the modified vector
- The resulting construct, pAG4, was tested by comparing luciferase expression (measured as luminescence) from the *E. coli araBAD* promoter, cloned in pAG4 and pUC18TminiTn7lux, and transposition of each into *P. aeruginosa*
- The results showed a reduced background and greater maximal expression for the pAG4-based reporter, yielding a > 16-fold increase in measured induction

Figure 1. Construction of pAG4. The region deleted initially is shown by the dashed line. Numbers indicate nucleotide positions (from GenBank AY962893.1). MCS* indicates the multiple cloning site of pUC18-mini-Tn7T-Gm-lux truncated at the *EcoRI* site.

Figure 2.

Effect of *lux* vector modification on reporter expression. The *E. coli* P_{araBAD} promoter including *araC* was cloned at the multiple cloning sites of pAG4 and pUC18-mini-Tn7T-Gm-lux vectors and transferred to the *P. aeruginosa* MPA01 chromosome by transposition. **A**, growth. Only control and the highest concentration of arabinose experiments are shown for each strain for clarity. Symbols/line styles: ●/solid, pAG4:: P_{araBAD} no arabinose; ○/dashed, pAG4:: P_{araBAD} 1.25 mg/ml arabinose; ■/solid, pUC18-mini-Tn7T-Gm-lux:: P_{araBAD} no arabinose; □/dashed, pUC18-mini-Tn7T-Gm-lux:: P_{araBAD} 1.25 mg/ml

arabinose. **B**, Luminescence time course. Symbols:●, pAG4:: P_{araBAD} 1.25 mg/ml arabinose; ■, pUC18-mini-Tn7T-Gm-lux:: P_{araBAD} 1.25 mg/ml arabinose; ○, pAG4:: P_{araBAD} 0.156 mg/ml arabinose; □, pUC18-mini-Tn7T-Gm-lux 0.156 mg/ml arabinose. RLU (relative light units). **C**, maximum luminescence versus inducer concentration. Symbols:●, pAG4:: P_{araBAD} ; ■, pUC18-mini-Tn7T-Gm-lux:: P_{araBAD} ; ○, pAG4 (empty); □, pUC18-mini-Tn7T-Gm-lux (empty). **D**, Induction ratio ($= \log_2[RLU_{induced}/RLU_{uninduced}]$) versus inducer concentration. Symbols:●, pAG4:: P_{araBAD} ; ■, pUC18-mini-Tn7T-Gm-lux:: P_{araBAD} ; Error bars represent standard deviations of triplicate experiments.

Table 1

Primers used.

Primer Name	Sequence
pUCTn7lux MCS Mfe L	ATCA ATT GATCTCGGCTTGAACGAATTG
pUCTn7lux MCS R	GAAGAATTGGGGATCAGCTTC
pJN105FW SpeI	TACA CTAGTT CGACGGTATCGATGCATAA
ParaBAD Eco R	AGAACTAGTGGATCCCCC
pAG4paraVerL	TCGCTAACCAAACCGGTAAC
pAG4ParaVerR	GCAACCGTAATTCGTTATTTCC
pUC18TluxVerR	AAAAAGTCATGCTCTTCTCTAATGC
P _{glmS-down}	GCACATCGGCGACGTGCTCTC
P _{glmS-up}	CTGTGCGACTGCTGGAGCTGA
P _{Tn7R}	CACAGCATAACTGGACTGATTTC
P _{Tn7L}	ATTAGCTTACGACGCTACACCC
pUCTn7mutFW	CAGAGCGATTTTGAAGCGAATTCGATCATGGATGG
pUCTn7mutRV	CCATCCATGATCGAATTCGCTTCAAAAGCGCTCTG