Construction of Mobilizable Mini-Tn7 Vectors for Bioluminescent Detection of Gram-Negative Bacteria and Single-Copy Promoter *lux* Reporter Analysis

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We describe the construction of mini-Tn7-based broad-host-range vectors encoding *lux* genes as bioluminescent reporters. These constructs can be mobilized into the desired host(s) by conjugation for chromosomal mini-Tn7-*lux* integration and are useful for localization of bacteria during infections or for characterizing regulation of promoters of interest in Gram-negative bacteria.

The *lux* bioluminescent reporter genes are useful for quantifying and characterizing bacterial gene expression or pinpointing the location of bacteria within *in vivo* infection models (1). Expression of the luciferase genes (*luxCDABE*) from *Photobacterium luminescens*, commonly referred to as the *lux* reporter, results in readily detectable bioluminescence (2). The light produced by luciferase is emitted at 490 to 500 nm and can be easily detected by several analytical platforms, such as luminometers, film exposure, or digital cameras. In this report, we describe the assembly of mobilizable *lux* reporter plasmid constructs capable of being transferred to the strain of interest by conjugation to integrate the mini-Tn7-*lux* elements into the chromosome of numerous Gram-negative bacteria. We provide experimental examples that highlight their utility and offer further suggestions for their application in future studies.

Construction of mini-Tn7-*lux* reporter vectors with *oriT* for mobilization and a trimethoprim resistance selection marker. Mini-Tn7 vectors have been shown to be useful in many bacteria (3–9) for shuttling constructs to a highly conserved site on the

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FIG 1 Genetic map of the key features of pUC18T-mini-Tn7T-*lux*-Tp. pUC18T-mini-Tn7T-*lux*-Tp contains two antibiotic resistance markers: *bla* (encoding β -lactamase) and *dhfr* (encoding trimethoprim-resistant dihydrofolate reductase). The *dhfr* marker is located on the transposable element and is used to select for chromosomal integration and can be removed via FLP-mediated recombination between the *FRT* sites. The P1 promoter (14) drives constitutive expression of the *luxCDABE* operon. The *oriT* allows mobilization of this vector into recipient strains by conjugation (11). The map was generated with CLC Workbench 6.

TABLE 1 Plasmids and PCR primers used in this study

Plasmid or primer	Accession no. and/or relevant characteristics or sequence ^a	Reference
Plasmid		
pUC18-mini-Tn7T-Gm- <i>lux</i>	AY962893; suicide vector for shuttling single copies of genes directly to the chromosome via a mini-Tn7 element; <i>aacC1</i> gene encoding gentamicin resistance marker on Tn7 element; P1 integron promoter driving expression of <i>luxCDABE</i> ; Amp ^r Gm ^r	4
pUC18T-mini-Tn7T-Tp	DQ493875; suicide vector for shuttling single copies of genes directly to the chromosome via a mini-Tn7 element; <i>dhfrIIIb</i> gene encoding trimethoprim resistance marker on Tn7 element; contains <i>oriT</i> for mobilization; Amp ^r Tp ^r	6
pUC18T-mini-Tn7T- <i>lux</i> -Tp	KC848883; suicide vector for shuttling single copies of genes directly to the chromosome via a mini-Tn7 element; <i>dhfrIIIb</i> gene encoding trimethoprim resistance marker on Tn7 element; contains <i>oriT</i> for mobilization; P1 integron promoter driving expression of <i>luxCDABE</i> ; Amp ^r Tp ^r	This study
pUC18T-mini-Tn7T- <i>lux</i> -Gm	KC848884; suicide vector for shuttling single copies of genes directly to the chromosome via a mini-Tn7 element; <i>aacC1</i> gene encoding gentamicin resistance marker on Tn7 element; contains <i>oriT</i> for mobilization; P1 integron promoter driving expression of <i>luxCDABE</i> ; Amp ^r Gm ^r	This study
pUC18T-mini-Tn7T- <i>PalgU</i> - <i>lux</i> -Tp	pUC18T-mini-Tn7T- <i>lux</i> -Tp with the P1 promoter replaced with the <i>PalgU</i> promoter region of <i>P. aeruginosa</i> strain PAO1; Amp ^r Tp ^r	This study
pUC18T-mini-Tn7T- <i>PalgD</i> - <i>lux</i> -Tp	pUC18T-mini-Tn7T- <i>lux</i> -Tp with the P1 promoter replaced with the <i>PalgD</i> promoter region of <i>P. aeruginosa</i> strain PAO1; Amp ^r Tp ^r	This study
pTNS3	Helper plasmid encoding the Tn7 site-specific transposition pathway; Amp ^r	19
pRK2013	Helper plasmid for conjugation; Kan ^r	20
PCR primers		
BamHI- <i>PalgU</i> -F	5'-CTAGGGATCCAAGTCGAGCCCTGCGACA	This study
EcoRI- <i>PalgU</i> -F	5'-CTAGGAATTCGAAAGCTCCTCTCGAAC	This study
BamHI- <i>PalgD</i> -F	5'-CTAGGGATCCGCTTTCCGACAAGCTCGCCGGCCT	This study
EcoRI- <i>PalgD</i> -R	5'-GATCGAATTCGCGATTACCTCGATTGTTTGTCG	This study
pLux seq F	5'-GAAGTTCCTATTCCGAAGTTCCT	This study
pLux seq R	5'-TTTTACCGGCAGATTCTAAAG	This study

^a Abbreviations: Amp, ampicillin; Gm, gentamicin; Kan, kanamycin; r, resistant; Tp, trimethoprim. Underlined sequences represent restriction enzyme sites.

chromosome downstream of the *glmS* gene(s) (4). A mini-Tn7 vector carrying *lux* driven by a constitutive promoter has been constructed and is known as pUC18-mini-Tn7T-*lux*-Gm (6). To permit conjugation of the plasmid into recipient cells, an *oriT*-containing fragment from the backbone of pUC18T-mini-Tn7T was released by digestion with NarI and StuI and ligated with a NarI-StuI fragment of pUC18-mini-Tn7T-Gm-*lux* to create the new vector pUC18T-mini-Tn7T-*lux*-Gm (see Fig. S1 in the supplemental material). Many bacterial mutant strains, such as those in the *Pseudomonas aeruginosa* PA14 nonredundant mutant library (10), were constructed with a gentamicin resistance marker. In order to make this vector compatible with a broader range of bacteria, we replaced the gentamicin resistance cassette (*aacC1*) with a trimethoprim resistance marker. To accomplish this, the XbaI-*FRT*-*aacC1*-*FRT*-XbaI fragment was deleted from pUC18T-mini-Tn7T-*lux*-Gm and replaced with the XbaI-*FRT*-*dhfrIIIb*-*FRT*-XbaI fragment from pUC18T-mini-Tn7T-Tp, forming pUC18T-mini-Tn7T-*lux*-Tp (Fig. 1). In order to integrate the mini-Tn7-*lux* elements contained in this and related constructs into the chromosome, these delivery vectors can be transferred by conjugation into recipient strains along with helper plasmids (pRK2013 and pTNS3; Table 1), as previously described (11). Alternatively, the delivery plasmid can be (i) transformed into an *Escherichia coli* mobilizer strain with chromosomally integrated transfer functions (e.g., RHO3, S17-1, or SM10) and conjugally cotransferred into the target bacterium containing the pTNS3 helper plasmid (12) or (ii) codelivered into the target bacterium by coelectroporation of the mini-Tn7-*lux* delivery plasmid and pTNS3 (6, 13).

Cloning of promoter *lux* fusions for transcriptional activity analysis. Both pUC18T-mini-Tn7T-*lux*-Tp and pUC18T-mini-Tn7T-*lux*-Gm contain the robust P1 integron promoter (14) which drives constitutive *luxCDABE* gene expression. The P1 integron promoter region has two sigma⁷⁰-dependent promoters; similar constructs with the P1 promoter have proven to be useful for bioluminescent localization studies (15). In addition, promoter *lux* fusions are useful for determining *in vitro* and *in vivo* promoter regulation, including the conditions that affect activity, and for basic characterization of promoter regions (2). To fuse a promoter of interest into the pUC18T-mini-Tn7T-*lux* vectors, BamHI, EcoRI, and PstI restriction sites can be used to remove the P1 integron promoter and replace it with a promoter of interest. We attempted to replace the P1 integron promoter region with a multiple cloning site but noticed instability of the plasmid during cloning and found it easier to delete the P1 promoter region by restriction enzyme digestion and replace it with the promoter of interest. The resulting construct was then able to be used to perform a variety of experiments aimed at characterizing factors governing gene expression from the cloned promoter region.

***In vitro* analysis of promoter activity with the *lux* reporter constructs.** *P. aeruginosa* is a Gram-negative opportunistic pathogen that secretes an exopolysaccharide known as alginate to promote survival in the lungs of cystic fibrosis patients. AlgU is the master regulator of alginate biosynthesis and is an alternative sigma factor that is required for the expression of the *algD* alginate biosynthetic operon. *PalgU* and *PalgD* are important regulated promoters that have been extensively studied (16). DNA fragments containing the *PalgU* and *PalgD* promoters were amplified

FIG 2 Methods of detection and various uses of bioluminescent *P. aeruginosa*. (A) The four *P. aeruginosa* strains were cultured on PIA at 37°C for 24 h. Using the Adobe action file to generate overlays (see Fig. S2 in the supplemental material), the various strains can be identified by bioluminescent intensity from images captured with a Bio-Rad Chemidoc XRS+ Gel Doc. (B) The same strains were mixed, plated on PIA, and grown at 37°C for 24 h. Each of the various reporter strains can easily be identified by its differential bioluminescence. (C) The strains were cultured overnight at 37°C in PIB, diluted 1:100, and inoculated into a 96-well plate with 100 μ l PIB. Optical density and bioluminescence were measured with a PerkinElmer Victor³ luminometer from 0 to 22 h. The data are represented in counts per second (CPS)/optical density (OD) at 550 nm. (D) Strains PAO1 with *PalgU-lux* and PAO1 with *PalgD-lux* were cultured on PIA and PIAAMV for 24 h at 37°C, cells were collected in phosphate-buffered saline (PBS), and the bioluminescence of the strains was measured in the Victor³ luminometer.

from strain PAO1 and directionally ligated into pUC18T-mini-Tn7T-*lux*-Tp to drive *lux* expression, and the respective mini-Tn7 elements were transferred to the chromosome of PAO1 as previously described (11). PAO1 derivatives with *P1-lux*, *PalgU-lux*, or *PalgD-lux* integrated into the chromosome were grown on *Pseudomonas* isolation agar (PIA) (Fig. 2A and B). A Bio-Rad Chemidoc XRS+ Gel Doc was used to detect bioluminescence. Standard gel imaging systems with chemiluminescence detection capabilities can be utilized to take white light and bioluminescence images that can then be overlaid with an Adobe Photoshop action file (<http://tinyurl.com/clzy83o>). This action file can be used to generate overlays following the protocol described in Fig. S2 in the supplemental material to identify clones with higher or lower bioluminescence production in the environment being tested (Fig. 2A and B). Using densitometry measurements (data not shown), each of the clones can be identified when they are mixed together and plated (Fig. 2B). Since PAO1 is nonmucoid on PIA, it was expected the *PalgD-lux* activity would be low on PIA (Fig. 2A). A PerkinElmer Victor³ luminescence plate reader was used to measure bioluminescence during growth. In addition to luminometer-based measurements, simple exposure to photographic film for a few seconds is sufficient to effectively visualize bioluminescence (data not shown). As shown in Fig. 2C, the activities of

PAO1 with *P1-lux*, *PalgU-lux*, and *PalgD-lux* were measured in *Pseudomonas* isolation broth (PIB). Bioluminescence was the highest for the *P1-lux* reporter during log-phase growth (Fig. 2C). We would predict the *PalgU* promoter activity to be higher than that of *PalgD* in PIB because PIB does not cause alginate overproduction (Fig. 2C). PIA with ammonium metavanadate (PIAAMV) is a medium that induces alginate overproduction by *P. aeruginosa* and activates transcription from both *PalgU* and *PalgD* (17). The *PalgU* and *PalgD* reporter strains were grown on PIA and on PIAAMV, cells were collected in phosphate-buffered saline, samples were diluted to the same optical density, and then the bioluminescence was measured by a luminometer. As shown previously with promoter-*lacZ* fusions (17), compared to PIA results, growth on PIAAMV increases *PalgU* and *PalgD* promoter activity (Fig. 2D).

In vivo analysis of promoter activity with the *lux* reporter constructs. To illustrate how the *lux* constructs can be used for localization of bacteria and *in vivo* promoter analysis, PAO1, PAO1-*P1-lux*, PAO1-*PalgU-lux*, and PAO1-*PalgD-lux* strains were used to infect lettuce (18). *P. aeruginosa* was grown in PIB at 37°C for 18 h and washed with sterile 10 mM MgSO₄. Fresh romaine lettuce leaves were washed with 0.1% (vol/vol) bleach and rinsed with sterile distilled water, and then the midrib was inocu-

FIG 3 *In vivo* bioluminescent detection of *P. aeruginosa* infecting lettuce and mice. (A) Image of *P. aeruginosa*-infected lettuce at 24 h postinoculation with PAO1 derivatives. (B) Bioluminescent detection of the PAO1 P1-*lux* reporter strain in a BALB/c mouse. The image was exposed for 10 s and captured by a Xenogen IVIS 100 imaging system.

lated with 10^6 CFU of each strain. A Bio-Rad Chemidoc XRS+ Gel Doc was used to detect bioluminescence of *P. aeruginosa* strains 24 h after infection. While the diameters of the soft rot area at the site of inoculation for all the strains were nearly identical to the diameter at the site of the uninfecting inoculation, the intensity of light emission for PAO1-P1-*lux* was the highest (Fig. 3A). *PalgU* and *PalgD* were also detected, albeit at lower levels, as expected. Comparable images were obtained with the Xenogen IVIS 100 imaging system (data not shown), but an imaging system of such ultrahigh sensitivity is not required for all applications. These promoter-*lux* constructs can also be used for *in vivo* imaging in other model infection systems, such as mice (Fig. 3B). Eight-week-old BALB/c mice were infected with 4×10^7 CFU of PAO1 P1-*lux* by intranasal administration. After 24 h, mice were anesthetized with isoflurane and images were captured by 10-s exposures with a Xenogen IVIS 100 imaging system (Fig. 3B). PAO1 P1-*lux* was clearly localized in the nares and lung of the mice shown in the representative image, indicating the utility of these constructs for *in vivo* bioluminescence imaging. All animal experiments were performed following protocols approved by the University of Virginia Animal Care and Use Committee.

Summary. In this report, we have described construction of two mobilizable mini-Tn7-based chromosomal integration vectors for luciferase tagging and promoter analysis using *lux* fusions. We showed several examples illustrating the utility of these vectors and imaging of the resultant bioluminescent bacteria. To discover novel regulators, a promoter of interest could be fused with the *lux* genes and then the strain could be submitted to transposon mutagenesis. Bioluminescent screening for resulting mutants with higher or lower promoter activity could identify novel regulators. Mini-Tn7-based vectors can be used in many Gram-negative bacteria, and we anticipate that these vectors will be of great utility for gene expression analyses in various model systems.

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