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 Photorhabdus 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 chromosome. In this study, we describe the construction of mobilizable mini-Tn7-lux vectors, which can be used for chromosomal integration and for localization of bacteria during infections. The mini-Tn7 vectors contain 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 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**

<table>
<thead>
<tr>
<th>Plasmid or primer</th>
<th>Accession no. and/or relevant characteristics or sequence*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC18-mini-Tn7T-Gm-lux</td>
<td>pUC18-mini-Tn7T-Gm; suicide vector for shuttling single copies of genes directly to the chromosome via a mini-Tn7 element; aacC1 gene encoding gentamicin resistance marker on Tn7 element; P1 integrase promoter driving expression of luxCDABE; Amp' Gm'</td>
<td>4</td>
</tr>
<tr>
<td>pUC18T-mini-Tn7T-Tp</td>
<td>DG493875; suicide vector for shuttling single copies of genes directly to the chromosome via a mini-Tn7 element; dhfrIIb gene encoding trimethoprim resistance marker on Tn7 element; contains oriT for mobilization; Amp' Tp'</td>
<td>6</td>
</tr>
<tr>
<td>pUC18T-mini-Tn7T-lux-Tp</td>
<td>KC848883; suicide vector for shuttling single copies of genes directly to the chromosome via a mini-Tn7 element; dhfrIIb gene encoding trimethoprim resistance marker on Tn7 element; contains oriT for mobilization; P1 integrase promoter driving expression of luxCDABE; Amp' Tp'</td>
<td>This study</td>
</tr>
<tr>
<td>pUC18T-mini-Tn7T-lux-Gm</td>
<td>KC848884; suicide vector for shuttling single copies of genes directly to the chromosome via a mini-Tn7 element; aacC1 gene encoding gentamicin resistance marker on Tn7 element; contains oriT for mobilization; P1 integrase promoter driving expression of luxCDABE; Amp' Gm'</td>
<td>This study</td>
</tr>
<tr>
<td>pUC18T-mini-Tn7T-PalgU-lux-Tp</td>
<td>pUC18T-mini-Tn7T-lux-Tp with the P1 promoter replaced with the PalgU promoter region of <em>P. aeruginosa</em> strain PAO1; Amp' Tp'</td>
<td>This study</td>
</tr>
<tr>
<td>pUC18T-mini-Tn7T-PalgD-lux-Tp</td>
<td>pUC18T-mini-Tn7T-lux-Tp with the P1 promoter replaced with the PalgD promoter region of <em>P. aeruginosa</em> strain PAO1; Amp' Tp'</td>
<td>This study</td>
</tr>
<tr>
<td>pTNS3</td>
<td>Helper plasmid encoding the Tn7 site-specific transposition pathway; Amp'</td>
<td>19</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Helper plasmid for conjugation; Kan'</td>
<td>20</td>
</tr>
</tbody>
</table>

**PCR primers**

| BamHI-PalgU-F          | 5'-CTAGGGATCCAAATTCGAGCCCTGCGGACA | This study |
| BamHI-PalgD-F          | 5'-CTAGGGATCCAGTCCATTCCCTGCGAACC | This study |
| EcoRI-PalgU-F          | 5'-CTAGGAATTGCGAAAGCTCCTTGCGAAC  | This study |
| EcoRI-PalgD-R          | 5'-GATCGGAATTCCGATCCATTCCGTCAATGTGGTTCG | This study |
| plux seq F             | 5'-GAAGTTCTCATTTCCGAGTTCTCT    | This study |
| plux seq R             | 5'-TTTTACCGGAGATTTCTAAG       | This study |

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

The 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 integrase promoter (14) which drives constitutive luxCDABE gene expression. The P1 integrase promoter region has two sigma27-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 integrase promoter and replace it with a promoter of interest. We attempted to replace the P1 integrase 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.

chromosome downstream of the glmS gene(s) (4). A mini-Tn 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 Stul and ligated with a NarI-Stul fragment of pUC18-mini-Tn7T-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-Gm and replaced with the XbaI-FRT-dhfrIIb-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., RH30, S17-1, or SM10) and conjuga-tely provided into the target bacterium by coelectroporation of the mini-Tn7-lux delivery plasmid and pTNS3 (6, 13).
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/ HDR1001 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 (PBS), and the bioluminescence of the strains was measured in the Victor3 luminometer.

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-
labeled with $10^6$ CFU of each strain. A Bio-Rad Chemidoc XRS+ Gel Doc was used to detect bioluminescence of \textit{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 uninfected inoculation, the intensity of light emission for PAO1-P1-\textit{lux} was the highest (Fig. 3A). \textit{PalgU} and \textit{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-\textit{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 $4 \times 10^7$ CFU of PAO1 P1-\textit{lux} by intranasal administration. After 24 h, mice were anesthetized with isoflu- rane and images were captured by 10-s exposures with a Xenogen IVIS 100 imaging system (Fig. 3B). PAO1 P1-\textit{lux} was clearly local- ized in the nares and lung of the mice shown in the representative image, indicating the utility of these constructs for in vivo biolu- minescence imaging. All animal experiments were performed fol- lowing protocols approved by the University of Virginia Animal Care and Use Committee.

**Summary.** In this report, we have described construction of two mobilizable mini-Tn\textsubscript{7}-based chromosomal integration vec- tors for luciferase tagging and promoter analysis using \textit{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 \textit{lux} genes and then the strain could be submitted to transposon mu- tagenesis. Bioluminescent screening for resulting mutants with imaging in other model systems was 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-\textit{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 $4 \times 10^7$ CFU of PAO1 P1-\textit{lux} by intranasal administration. After 24 h, mice were anesthetized with isoflu- rane and images were captured by 10-s exposures with a Xenogen IVIS 100 imaging system (Fig. 3B). PAO1 P1-\textit{lux} was clearly local- ized in the nares and lung of the mice shown in the representative image, indicating the utility of these constructs for in vivo biolu- minescence imaging. All animal experiments were performed fol- lowing protocols approved by the University of Virginia Animal Care and Use Committee.

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**References**


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


