Abstract  Fusions of genetic regulatory elements with reporter genes have long been used as tools for monitoring gene expression and have become a major component in synthetic gene circuit implementation. A major limitation of many of these systems is the relatively long half-life of the reporter protein(s), which prevents monitoring both the initiation and the termination of transcription in real-time. Furthermore, when used as components in synthetic gene circuits, the long time constants associated with reporter protein decay may significantly degrade circuit performance. In this study, short half-life variants of LuxA and LuxB from Photorhabdus luminescens were constructed in Escherichia coli by inclusion of an 11-amino acid carboxy-terminal tag that is recognized by endogenous tail-specific proteases. Results indicated that the addition of the C-terminal tag affected the functional half-life of the holoenzyme when the tag was added to luxA or to both luxA and luxB, but modification of luxB alone did not have a significant effect. In addition, it was also found that alteration of the terminal three amino acid residues of the carboxy-terminal tag fused to LuxA generated variants with half-lives of intermediate length in a manner similar to that reported for GFP. This report is the first instance of the C-terminal tagging approach for the regulation of protein half-life to be applied to an enzyme or monomer of a multi-subunit enzyme complex and will extend the utility of the bacterial luciferase reporter genes for the monitoring of dynamic changes in gene expression.

Keywords  Luciferase · Reporter genes · Gene expression · Lux · Destabilized protein · ssrA · Photorhabdus luminescens

Introduction

Reporter genes are transcriptional fusions of genetic regulatory elements to reporter proteins that provide readily detectable signal outputs. Such reporter proteins include LacZ, chloramphenicol acetyltransferase, β-lactamase (Zlokarnik et al. 1998), firefly luciferase (luc), bacterial luciferase (lux), and fluorescent proteins such as those derived from the green fluorescent protein of Aequorea victoria (e.g. GFP, EGFP, YFP) (Chalfie et al. 1994; Cormack et al. 1996; Andersen et al. 1998; Miyawaki et al. 1997; also reviewed in van der Meer et al. 2004). These gene fusions have been used in detecting xenobiotic compounds (King et al. 1990; DiGrazia et al. 1991), monitoring performance of synthetic gene circuits (Elowitz and Leibler 2000; Hasty et al. 2002; Gardner et al. 2000), in genome-wide expression arrays (Van Dyk et al. 2001), as well as in in vivo imaging studies (Xiong et al. 2005; Kadurugamuwa et al. 2003; Kuklin et al. 2003; Doyle et al. 2004).
While these reporter proteins have numerous applications, all except the bacterial luciferase system require the additional input of reactants or UV light. The bioluminescent gene cassette comprising the lux system was originally isolated from *Vibrio fischeri* (Engebret et al. 1983). Later, more thermally stable versions of lux were isolated from *Photorhabdus* (*Xenorhabdus*) *luminescens* and other species (Schmidt et al. 1989; Szittner and Meighen 1990). In these systems, the heterodimeric bacterial luciferase comprised of LuxAB oxidizes an aliphatic aldehyde substrate and FMNH$_2$ in the presence of diatomic oxygen to release a photon of light at a wavelength of ~490 nm (reviewed in Meighen (1991)). The remainder of the cassette consisting of the luxC, D and E genes, encode proteins that are responsible for generating and recycling the aldehyde substrate for the reaction.

Another major problem with most reporter systems are the relatively long half-lives of the reporter proteins, which complicate the detection and monitoring of dynamic changes in gene regulation at the transcriptional level. To address these issues, modifications to the protein coding sequences of some of these reporter systems have been made to include amino acids sequences that are recognized by host protein degradation machinery resulting in more rapid turnover of the reporter protein (Andersen et al. 1998; Mateus and Avery 2000; Li et al. 1998; Triccas et al. 2002). Similar tactics have been employed in the engineering of synthetic gene circuits where dynamic changes in transcription, or in the case of protein half-lives of intermediate lengths, the heterodimeric bacterial luciferase comprised of LuxAB oxidizes an aliphatic aldehyde substrate and FMNH$_2$ in the presence of diatomic oxygen to release a photon of light at a wavelength of ~490 nm (reviewed in Meighen (1991)). The remainder of the cassette consisting of the luxC, D and E genes, encode proteins that are responsible for generating and recycling the aldehyde substrate for the reaction.

Finally, the carboxy-terminal tags were cloned into an existing plasmid in a manner that should facilitate retrofitting of other P. *luminescens* lux-based reporters for the monitoring of rapid changes in transcription, or inclusion into synthetic gene circuits where dynamic signal performance is critical.

**Materials and methods**

**Genetic constructs**

Wild type and modified variants of the luxA and luxB genes were amplified by PCR from a vector containing the *P. luminescens* lux CDABE operon (pCDABE) using the primers listed in Table 1. Abbreviations for the tag (e.g., -aav) are indicated in the name of the construct after the gene being affected. Wild type luxAB and luxABAav were amplified by PCR, gel purified, and cloned into the TOPO-TA cloning vector pCR2.1 (Invitrogen) per the manufacturer's instructions. For luxA variants, luxBAav, and wild-type luxB, the genes were independently amplified and cloned. The intervening sequence upstream of the ribosomal binding site between luxA and luxB was modified to create a unique XmaI site and facilitate subsequent construction. The luxA variants were digested overnight with XmaI and XbaI and ligated upstream of the luxB or luxBAav genes in pCR2.1. The wild type luxAB and the newly assembled variants were then each double digested overnight from their respective TA-cloning vectors with *BamHI* and *KpnI*, gel purified, and directionally cloned in place.
of the *GFPasv* gene of the repressilator reporter plasmid (Elowitz and Leibler 2000). For insertion of the tagged *luxA* into an existing full *lux* cassette, the appropriate primary construct was subsequently digested internal to both *luxA* (AgeI) and *luxB* (BfuAI) and the tag-bearing fragment was directionally cloned into the similarly digested pCDABE. Cloning reactions were conducted at room temperature for 10 min and included: 100 ng vector DNA, a 2:1 molar ratio of insert to vector DNA, 4 U of T4 DNA ligase (Fisher Scientific, Pittsburgh, PA) and 2 · Ligation rapid ligation buffer (Promega, Madison, WI) in 25–35 l reactions. Restriction enzymes were purchased from New England Biolabs (Beverly, MA), and all digestions were conducted using 5 U of each enzyme and incubated overnight at 37°C except in the case of BfuAI, in which digestion was incubated for 4 h at 50°C, followed by transfer to 37°C and the addition of AgeI.

**Strains and growth conditions**

Strains used in this study are listed in Table 2. Luria-Bertani media (Sambrook et al. 1989) was used for all experiments with 50 µg/ml kanamycin as necessary, and incubated at 37°C with shaking at 100 r.p.m. when appropriate.

**Bioluminescence assays**

Overnight cultures of the four variants of luxAB and a negative control carrying the parent vector with the gene for destabilized green fluorescent protein, Gfpasv, were transferred into triplicate tubes of fresh LB medium containing 50 µg/ml kanamycin. Cultures were then grown to an optical density at 546 nm (OD546) of ~0.6. Initial bioluminescence measurements were made at t = 0 as described below. Subsequently, rifampicin (150 µg/ml) and tetracycline (30 µg/ml) were added to all cultures to block transcription and translation, respectively. For bioluminescence measurements, 1-ml samples were taken immediately (t = 0) and at 30 min intervals thereafter and assayed for optical density at 546 nm (OD546) and bioluminescence. OD546 was measured using a Beckman DU-640B spectrophotometer. Bioluminescence was assayed by first adding 10 l of N-decanal (Sigma, St. Louis, MO) to the 1-ml samples, inverting 10 times, and measuring light with a SDI Deltatox handheld luminometer. Three light measurements were taken in rapid succession and these values were averaged for each sample. Error bars represent the standard deviations between the averages of three independent samples.

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**Table 1 Primers used in this study**

| Primer              | Restriction Site | Forward/Reverse | Nucleotide Sequence |
|---------------------|------------------|-----------------|--------------------|
| LuxA forward        | KpnI             | Start           | 5’-GGTACC GC ATG AAA TTT GGA AAT TTT TTG -3’ |
| LuxB forward        | XmaI             | Start           | 5’-CCC GG GCT AAG GAG AAA ATG AAA T –3’ |
| LuxB reverse        | BamHI            | Stop            | 5’-TTA GGCATCC TA TTA G GTA TAT TCC ATG TGG TAC TCC –3’ |
| LuxAsv reverse      | XmaI             | Stop            | 5’-TTA CCC GG GCT A AA TGC TGC AGC GTA GTT TTC GTC GTT TGC –3’ |
| LuxAaa reverse      | XmaI             | Stop            | 5’-TTA CCC GG GCT A CA TAA AGC TGC TAA TGC AGC GTA GTT TTC GTC GTT TGC –3’ |
| LuxBaav reverse     | BamHI            | Stop            | 5’-TTA GGCATCC TA TTA AAC TGG TGC AGC GTA GTT TTC GTC GTT TGC –3’ |

Unique restriction sites used in the final assembly are underlined. Start and stop codons and pertinent amino acids are indicated above the respective sequences.
Variants of luxA containing the -laa, -aav and -asv tags were chosen for further analysis. Plasmids containing the constructs were digested with AgeI and BfuAI, gel purified, and cloned as described above into the similarly treated pCDABE. The latter contains the entire P. luminescens lux operon under the control of the lac promoter in the multi-copy pCR-XL-TOPO vector (Invitrogen). Resulting plasmids were then transformed into E. coli TOP10 F’ cells (Invitrogen) for IPTG-inducible expression. For induction experiments, overnight cultures of cells grown in LB with 0.2 mM IPTG were diluted 1:5 in the same, fresh, preheated medium, and incubated at 37°C until reaching an OD600 of 0.15–0.2 (approximately 4 h). Cells were subsequently collected by centrifugation, washed with fresh M9 containing 100 mM leucine medium without inducer, and incubated in M9 + leucine medium at 37°C. One milliliter samples were removed every 20 min for bioluminescent measurements using a SDI Deltatox handheld luminometer. Three measurements were taken in rapid succession for each sample and their values were averaged, and three independent replicates were performed for each variant.

Results and discussion

Bioluminescence measurements of cells harboring plasmids containing the wild type luxAB genes of P. luminescens behind a constitutive promoter were compared to those of luxA and luxB variants containing carboxy-terminal tags designed to accelerate degradation of the protein(s) within the cell. Assays using constructs containing only luxA and luxB (i.e. which lack luxC, D, and E and hence are unable to produce the substrate) were conducted by adding antibiotics to block transcription and translation (rifampicin and tetracycline, respectively), followed by monitoring bioluminescence with the addition of aldehyde over time. As shown in Fig. 1, modification of the LuxB protein did not result in a decrease of bioluminescence. Bioluminescence in this variant was found to remain relatively constant similar to the wild type over the course of the experiments. Addition of the same -aav tag (encoding AANDE-NYAAAV) to the luxA gene resulted in a rapid decrease in bioluminescence after time t0. An even more dramatic decrease in bioluminescence was observed when both LuxA and LuxB included the -aav tag. Interestingly, the clone containing luxA-Baav (wild type luxA with modified luxB) appeared to produce more light than did the wild-type luxAB. Since this result was reproducible within the experimental parameters (Fig. 1) yet inconsistent with those generated in the absence of antibiotics (Table 3), it is believed that this result is an experimental artifact.

Table 2 Strains and plasmids used in this work

| Name | Properties | Source |
|------|------------|--------|
| E. coli TOP10 and TOP10F’ | Cloning and expression strains. Expression from the lac promoter in F’ cells is IPTG inducible | Invitrogen (Carlsbad, CA) |
| pCR2.1 | TOPO TA Cloning vector. AmR, KmR | Invitrogen |
| pCDABE | pCRXL (Invitrogen) containing PCR-amplified luxCDABE from Photobacterium luminescens | Gift from J. Sanseverino |
| pZE21-GFPasv | KmR; Plasmid containing short half-life GFPasv constitutively expressed from the Ptet01 promoter | Elowitz and Leibler |
| pAB | pZE21-GFPasv with the wild-type luxAB of P. luminescens cloned in place of GFPasv | This work |
| pABaav | Similar to pAB using the alternative tag AANDENYAAAV translationally fused to luxB by PCR | This work |
| pAaavBaav | Similar to pAB using the luxA and luxB genes each modified to include the gene sequence for the 11-amino acid carboxy-terminal tag AANDENYAAAV | This work |
| pAaavB | pAB containing the luxA gene modified to include the gene sequence of the 11-amino acid carboxy-terminal tag AANDENYAAAV, and the wild type luxB | This work |
| pAlaaB | Similar to pAaavB using the alternative tag AANDENYAAAV | This work |
| pAasvB | Similar to pAaavB using the alternative tag AANDENYAAASV | This work |
| pCDAasvBE | pCDABE containing the 3′ end of the modified luxA gene and the 5′ end of luxB from pAsvB | This work |
| pCDAaavBE | As in pCDAasvBE but using pAaavB | This work |
| pCDLaab | As in pCDAasvBE but using pAlaaB | This work |
In the unregulated case where enzyme was allowed to accumulate to a steady state level dependent upon the relative half-lives of their luciferases, mid-log cultures of pLuxAaavB produced approximately 100-fold less bioluminescence than the wild-type (Table 3). The double modified holoenzyme produced bioluminescence nearly 800-fold less than the wild type (Table 3).

To determine if the drop in bioluminescence output could be attributed solely to interference of the carboxy-terminal tag to enzymatic activity, two additional constructs were made with predicted half-lives greater (−asv encoding AANDENYAASV) and lesser (−laa encoding AANDENYALAA) than those predicted for −aav. Total bioluminescent output for all of these constructs is shown in Table 3.

Variants of luxA were subsequently cloned into a vector containing the entire lux operon by restriction digestion interior to each of the luxA and B genes and replacement of the wild type fragment with fragments containing the modified luxA gene. Results of experiments on luminescent decay following the removal of inducer (isopropyl-β-D-thiogalactopyranoside; IPTG) are shown in Fig. 2.

**Conclusions**

The results for variants containing the modified luxA gene are consistent with those previously reported by Anderson et al. (Andersen et al. 1998) for GFP, and indicate that the single modification of this monomer is sufficient to result in decreased functional half-life of the heterodimeric holoenzyme (Fig. 1 and Table 3). This modification may impact enzymatic activity of the holoenzyme since the carboxy-terminus of LuxA is thought to be involved in substrate binding (Valkova et al. 1999). However, bioluminescence outputs for all variants were readily detectable under our test conditions. Interestingly, modification of the LuxB monomer

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**Table 3** Comparison of total (arbitrary units) and relative (% w.t.) bioluminescence output from 1-ml samples of mid-log cultures (OD<sub>546</sub> = ~0.4) containing the indicated plasmids after addition of 10 µl N-decanal

| Plasmid     | Total bioluminescence | Std. dev. | % w.t. | Est. half-life |
|-------------|-----------------------|-----------|--------|---------------|
| pAaavBaav   | 2,510                 | 77.56     | 0.13   | N.D.          |
| pAlaaB      | 11,998                | 713.5     | 0.61   | 46            |
| pAaavB      | 17,057                | 1,524     | 0.87   | 48            |
| pAsvB       | 632,260               | 9,424     | 32.21  | 145           |
| pABaav      | 1,994,751             | 79,169    | 101.64 | > 240         |
| pAB         | 1,962,650             | 121,346   | 100.00 | > 240         |
| Negative control | 504                  | 42        | N.D.   | N.A.          |

Values for half-lives are in minutes. N.D. = not determined; N.A. = not applicable. Negative control consisted of the parent strain containing no plasmid.
alone neither decreased peak bioluminescence nor resulted in decay of bioluminescence over time when compared to the wild type. Modification of both luxA and luxB genes to include the -aav tag (luxAaavBaav), however, resulted in lower total bioluminescence than modification of luxA alone (Fig. 1). These results suggest that the carboxy-terminus of LuxB may be protected in the heteroduplex of the holoenzyme and inaccessible to cellular proteases, but its release following the degradation of LuxA may facilitate its subsequent degradation and account for the reduced bioluminescence of the double-modified variant.

Modification of protein half-lives in eukaryotic proteins by the carboxy-terminal additions of the PEST-rich region from the G1 cyclin Cln2 and the degradation domain of mouse ornithine decarboxylase have been used to generate destabilized variants of GFP in yeast and mammalian systems (Mateus and Avery 2000; Li et al. 1998), as well as firefly luciferase (Leclerc et al. 2000). Such an approach could also be applied to the luxA and luxB genes, which have been codon-optimized for expression in eukaryotic systems (Gupta et al. 2003; Patterson et al. 2005). Furthermore, the approach taken here in which the insertion of the luxA-tag-B fragment into an existing construct containing the entire P. luminescens lux cassette should be widely applicable to a number of existing lux-based reporter systems, and will allow relatively simple retrofitting of those constructs to generate short half-life variants for dynamic monitoring of changes in gene expression in reporter gene transcription, in vivo imaging, and synthetic biology applications.

Statement of competing interests MSA and GSS have applied for patents on the materials described here.

Author’s contributions MSA, JRW, and CSC carried out the molecular biology experiments and bioluminescence assays. MSA, GSS, and MLS conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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