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Note

A broad-host range dual-fluorescence reporter system for gene expression analysis in Gram-negative bacteria

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ABSTRACT

Fluorescence-based reporter systems are valuable tools for studying gene expression dynamics in living cells. Here we describe a dual-fluorescence reporter system carrying the red fluorescent marker mCherry and the blue fluorescent protein EBFP2 enabling the simultaneous analysis of two promoters in broad-host range autofluorescent Gram-negative bacteria.

Fluorescence techniques such as plasmid-mediated reporter systems are effective tools for investigating gene expression dynamics in living bacterial cells. The green fluorescent protein (GFP) isolated from the jellyfish *Aqeuorea victoria* is the most widely characterized and used fluorescent protein. Currently, several dual-fluorescence plasmid reporter systems based on red and green fluorescence are available for various applications in microbiology (Choe et al., 2005; Brzoska and Firth, 2013; Othman et al., 2013). However, the utility of GFP as a fluorescent marker is dependent on the host selected for analysis as many bacteria and other microbes (*e.g.* fungi) have natural spontaneous fluorescence referred to as autofluorescence that overlaps with the low emission of GFP. In the case of fluorescent pseudomonads, autofluorescence of the bacteria is due to the secretion of fluorescent siderophores also known as pyoverdines, used to chelate iron under iron-limited or low nutrient conditions (Neilands, 1995; Bultreys et al., 2001; Lamichhane and Varvaro, 2012). An array of alternative fluorescent proteins is currently available and, when selecting an appropriate marker several additional factors should be considered as reviewed by Shaner et al. (2005). For example, aside from autofluorescence, it is important to consider the excitation and emission profiles of fluorescent proteins to prevent spill over from other channels. Once an appropriate fluorophore is selected, the next step is choosing an appropriate plasmid system.

The Standard European Vector Architecture (SEVA) plasmids are modular broad-host range vectors designed to facilitate the swapping of various functional modules (*e.g.* antibiotic resistance markers, origins of replication) (Silva-Rocha et al., 2013). In a previous study, we have demonstrated the utility of reporter strains constructed using the single reporter vector pSEVA237R where, mCherry was fused to the promoter of *nunF*, a regulator of antifungal secondary metabolites, in order to study the expression dynamics of this gene in the biocontrol strain *Pseudomonas fluorescens* In5 in response to rhizosphere and hyphosphere-associated molecules and in co-culture with a pathogenic fungus (Hennessy et al., 2017a; Hennessy et al., 2017b). Expression of *nunF* was upregulated in response to hyphosphere-associated compounds (trehalose and glycerol) which also induced pyoverdine production. Furthermore, both bacterial and fungal-derived green fluorescent compounds were detected during the interaction of *P. fluorescens* In5 with the plant pathogen *Fusarium graminearum*. Expression of *nunF* was measured using a single fluorescence reporter system and was normalized by measuring cell biomass.

An advantage of using a dual fluorescence reporter over a single fluorescence system is the possibility for simultaneously analyzing two genes or alternatively it can serve as a tool similar to qPCR-based techniques where gene expression of a single gene is normalized to a reference gene, typically a housekeeping gene. Normalization and quantification of gene expression using housekeeping genes is a relatively simple and widely used method. Commonly used bacterial reference genes including in *Pseudomonas* species are *16S rRNA genes*, *rpoA*, *rpoD* and *gyrB* (Savli et al., 2003; Rocha et al., 2015; Chan et al., 2016). However, the commonly used reference genes are often subjected to regulation. For example, Alqarni et al. (2016) showed that 12 out of 13 housekeeping genes in *Pseudomonas aeruginosa* were differently regulated during carbon starvation, Tasara and Stephan (2007) documented that 4 out of 5 housekeeping genes were regulated in sixteen different *Listeria* strains, and Menzel and Gellert (1987) showed that *gyrA* and *gyrB* were regulated by coumermycin treatment. An alternative method for normalization of gene expression is based on

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targeting genomic DNA or cell density. However, in bacteria this is not ideal as replicating bacteria can contain significantly more copies of certain loci than non-replicating cells (Huggett et al., 2005). In addition, depending on growth conditions cells can differ in shape and size and therefore affect gene expression. Furthermore, RNA extraction procedures are typically not designed for DNA purification thus the final DNA yields obtained may be low and vary across samples (Huggett et al., 2005). In conclusion, when analyzing transcription activity using transcriptomics, qPCR or as here with fluorescent reporter strains care should be taken in selecting the optimal reference.

In this study, we assembled and tested a dual-fluorescence plasmid-based system expressing the red (mCherry) and blue (EPFP2) fluorescent proteins as a tool to investigate the response of a regulated *P. fluorescens* In5 promoter. The *nunF* promoter previously characterized was quantified relative to the reference housekeeping gyrase B subunit gene (*gyrB*) in the fluorescent bacterium *P. fluorescens* In5. When using quantitative real-time polymerase chain reaction (qRT-PCR) as a technique for mRNA quantification, expression values are normalized to an internal control typically a housekeeping gene. An important consideration when selecting a housekeeping gene is that expression of the reference gene is stable under all conditions tested. In order to select an appropriate housekeeping gene, we examined expression data of six housekeeping genes from a recently performed transcriptomics study on *P. fluorescens* In5 (Hennessy et al., 2017c). Of the six housekeeping genes investigated (*fabD*, *gyrA*, *gyrB*, *proC*, *rpoB* and *rpoD*) none of the genes showed significantly different expression levels across the three treatments tested ($P > 0.05$) (Supplementary Table S1). However, there were significant differences between expression levels of each gene across all treatments tested ($P < 0.001$) (Supplementary Table S1). The lowest relative expression was recorded for *gyrB* followed by *proC*. Compared to *gyrB*, the *gyrA* and *fabD* showed a 17-and 10-fold higher expression level respectively. The highest expression levels were recorded for *rpoB* and *rpoD*. In order to minimize the risk that expression and measurements of the housekeeper interfere with the measurements of the target gene, the *gyrB* housekeeper showing lowest expression values was selected as a reference gene for use in the dual reporter despite not showing the lowest variation across treatments. It has previously been reported that very high expression of fluorescent proteins can create stresses in the cell of study (Ganini et al., 2017). For example, GFP requires oxygen for maturation and consequently under low oxygen pressure, the fluorophore will compete with normal respiration for oxygen. This situation is worse if expression is high. There will also be other loads on the system of study and thus it is best to keep total fluorescent protein expression as low as possible to minimize the risk of studying artefacts (Ganini et al., 2017). It is important to validate studies based on fluorescent proteins with alternative methods and consequently interpret results carefully.

The dual fluorescent reporter plasmid pSEVA237RB was constructed by amplifying the blue fluorescent protein EBFP2 synthesized as a gene block (IDT) based on the sequence from the plasmid pBAD-EBFP2 described by Ai et al. (2007) and subsequently used as a template for PCR. The EBFP2 gene was then cloned by Gibson Assembly® (GA) (BioNordika) into *Avr*II-*Pac*I digested pSEVA237R (RK2-Km^R-mCherry) and the resulting dual reporter plasmid was named pSEVA237RB (Fig. 1). The promoter region located upstream of the start codon of the housekeeping *gyrB* gene (HM070426) was amplified from *P. fluorescens* In5 genomic DNA and cloned by GA into *Bam*HI-*Eco*R1 digested pSEVA237RB. The resulting plasmid was named pSEVA237RB::P_{gyrB}. The promoter region of the target gene *nunF* (WP_054049653) was amplified as above and cloned by GA into either *Xba*I-*Spe*I digested pSEVA237RB::P_{gyrB} generating the dual reporter strain *P. fluorescens* In5 harbouring the plasmid pSEVA237R::P_{nunF}B::P_{gyrB}. All plasmids were confirmed by restriction digest analysis and Sanger sequencing (GATC) as described above prior to transformation. The empty vector control plasmid pSEVA237RB and the tester plasmid pSEVA237R::P_{nunF}B::P_{gyrB} (P_{nunF}::*mCherry*, P_{gyrB}::*EBFP2*) were electrotransformed into *P. fluorescens* In5 as previously described (Michelsen et al., 2015). The resulting reporter strains used in this study are listed in Table 1 and the primer sequences for plasmid assembly are listed in Supplementary Table S2. For monitoring *nunF* gene expression *in vivo* compared to the housekeeper gene *gyrB, P. fluorescens* In5 reporter strains harbouring either pSEVA237RB (control), pSEVA237RB::P_{gyrB} (control) and pSEVA237R::P_{nunF} B::P_{gyrB} (tester) were grown overnight in full strength defined Fusarium medium (DFM) (Frandsen et al., 2006) minimal media (DFM) supplemented with 0.5% wv^{-1} glucose and 25 μg ml^{-1} kanamycin with shaking 200 rpm at 28 °C. Cells were washed twice with 0.9% wv^{-1} NaCl and resuspended to an OD_{600nm} = 0.1 and 20 μl was added to a 96-well microtiter plate, together with 180 μl of DFM with 0.05% wv^{-1} of glucose or replaced with0.05% wv^{-1} glycerol. The BMG LABTECH MARS (Omega V5.11) scripting function was used to multiplex protocols for simultaneously measuring, in the following order; mCherry (red) fluorescence ($F_{584/620\text{-}10}$), GFP (green) or autofluorescence ($F_{485\text{-}12/520}$), EBFP2 (blue) fluorescence ($F_{355/400}$) and growth (biomass) (A_{600nm}) every hour for 48 h at 28 °C in a FLUOstar Omega Microplate Reader (BMG LABTECH). Gene expression analysis of promoter-reporter gene fusions was performed using biological triplicates.

Previously we reported the upregulation of *nunF* expression in response to glucose but also glycerol, an indicator of the hyphosphere (Hennessy et al., 2017b). To test the dual-fluorescence reporter system as a tool to quantify gene expression, the response of *nunF* to glucose (control) and glycerol (fungal associated carbon source) was examined. Multiple fluorescence signals (mCherry, EBFP2 and green fluorescence in a GFP channel) cell density was recorded (Fig. 2 and Figs. S1–S3). In accordance with previous findings, the highest mCherry signal was observed for glycerol followed by glucose and cellobiose (Fig. 2B). The presence of the reporter plasmids did not negatively impact growth of the reporter strains (Figs. S1–S3). Interestingly, variation in green fluorescence was recorded between the three plasmids tested indicating that the constructs affect pyoverdine production (Figs. S1–S3). Relative blue fluorescence (EBFP2) was equivalent for the control (pSEVA237RB::P_{gyrB}) and tester (pSEVA237R::P_{nunF}B::P_{gyrB}) reporter constructs as predicted. As observed from the transcriptomics data, *gyrB* expression was low (Fig. 2A). However, although we used transcriptomics data in order to select the optimal reporter promotor (*gyrB*), this promotor might not be the best as the promotor seemed to be regulated by cellobiose (Fig. 2A–B).

In the present study, we constructed a dual-fluorescence reporter plasmid (pSEVA237RB) containing the blue fluorescent protein (EBFP2) and the red fluorophore mCherry. The advantages with this dual fluorescence reporter plasmid are (i) the possibility to assay two promoters simultaneously on a single plasmid without introducing bias of plasmid copy numbers that would result if the promoters were on different plasmids; (ii) no use of GFP since the GFP signal and fluorescent compounds formed by especially fluorescent pseudomonads have similar fluorescence and therefore interfere (iii) modular structure enables exchange of modules (*e.g.* fluorescence markers); (iv) serve as qPCR-like tool where expression of a tester gene is normalized to a housekeeper gene instead of growth; (v) function in a broad range of bacteria. The reporter system here described thus facilitates the quantification of gene expression normalized to a housekeeper gene or alternatively can be used to study two promoters or genes of interest simultaneously. As it is based on the SEVA plasmid system, the construct functions in diverse prokaryotes while the modular structure enables the easy exchange of parts (*e.g.* fluorescence reporters or promoter/gene of interest fragments). This flexibility of fluorescent proteins is particularly useful for studying organisms where autofluorescence can hamper analysis *e.g.* study of microbial interactions or microbial activity in soil systems.

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