Novel fluorescent reporters for studying pathogen-host interactions

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Introduction

Fluorescent proteins, such as green fluorescent protein (GFP), have diverse applications in the basic and applied sciences. While GFP has been frequently used in eukaryotic systems, its applications have been limited in microorganisms due to a lack of broad-range molecular tools. In this study, we have developed a vector to express GFP in pathogenic bacteria for use in bacterial pathogenesis and pathogen-host studies. A shuttle vector encoding the GFP variant *mut3*[1] (pUCP18-MCS*gfpmut3*) was generated and successfully transformed into various Gram-negative opportunistic pathogens from the ATCC collection, including: *Escherichia coli* (ATCC® 25922™), *Salmonella enterica* (ATCC® 14028™), *Shigella flexneri* (ATCC® 12022™), *Pseudomonas aeruginosa* (ATCC® 10145™), and the *P. aeruginosa* type strain PAO1 (ATCC® 15692™). *P. aeruginosa* was used as a model to test the characteristics of the vector and sensitivity of detection using a fluorescence plate reader, microscopy, flow cytometry, and *in vivo* imaging systems.

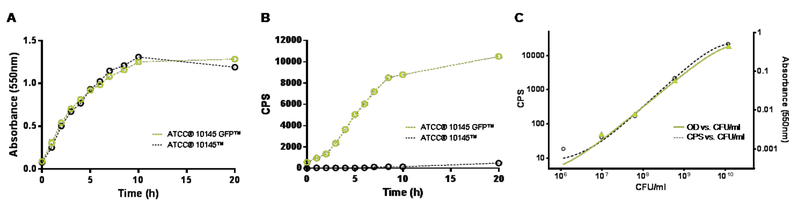
ATCC number*	Species	Reporter
ATCC® 10145GFP™	*Pseudomonas aeruginosa*	GFP
ATCC® 15692GFP™	*Pseudomonas aeruginosa*	GFP
ATCC® 25922GFP™	*Escherichia coli*	GFP
ATCC® 14028GFP™	*Salmonella typhimurium*	GFP
ATCC® 12022GFP™	*Shigella flexneri*	GFP

**Available Summer 2014

Results

The expression of the *gfpmut3* gene was monitored during growth (Figure 1). GFPmut3 did not alter bacterial growth in either *P. aeruginosa* (Figure 1A) or *E. coli* (data not shown). Fluorescence was easily detected and quantified using a fluorescence microplate reader (Figure 1B). A linear correlation was observed between fluorescence and colony forming units (cfu) (R^2=0.998) or optical density (R^2=0.999) at concentrations ranging from 10^6 to 10^{10} cfu/mL (Figure 1C).

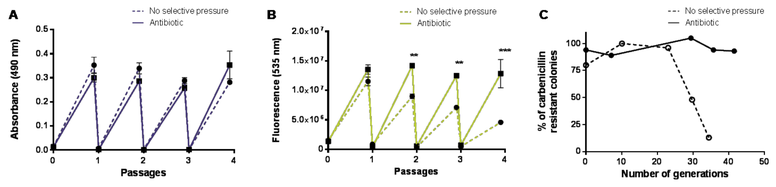
Figure 1: Expression of GFPmut3 does not affect bacterial fitness and can be used for bacterial quantification



P. aeruginosa ATCC® 10145™ (with empty vector control; black) and ATCC® 10145GFP™ (green) were grown at 37°C in Lysogeny broth (LB) with 300 µg/mL carbenicillin under constant shaking. A) Absorbance at 550 nm and B) fluorescence expressed as counts per second (CPS) (Ex. = 490 nm, Em. = 535 nm) were determined at regular intervals (VICTOR™ *X*3 Multilabel Plate Reader, PerkinElmer). C) Colony forming units (CFU) were determined by plating appropriate dilutions on Lysogeny Agar (LA) plates and were compared to CPS (black) and absorbance (green) using a non-linear regression.

The stability of the plasmid was tested in *P. aeruginosa* by passaging the bacterium in the absence of antibiotic pressure (Figure 2A and B). The plasmid was stable for 3 successive passages and for a minimum of 20 generations (Figure 2C) in *P. aeruginosa* ATCC® 10145GFP™.

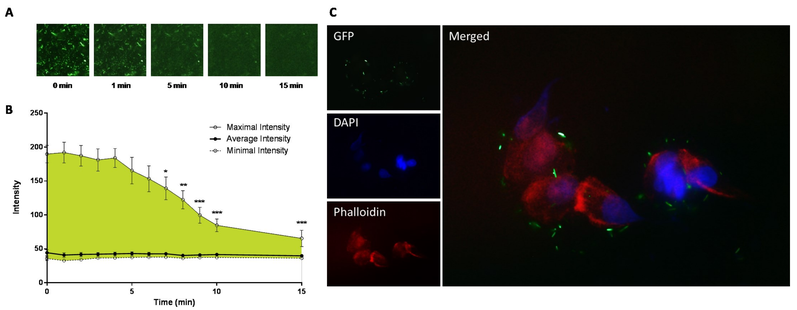
Figure 2: Plasmid stability in *P. aeruginosa* ATCC® 10145GFP™



P. aeruginosa ATCC® 10145GFP™ was grown at 37°C in Lysogeny broth (LB) with 300 µg/mL carbenicillin (continuous line), or in the absence of antibiotic pressure (dashed line), under constant shaking. Cultures were passaged every 24 hrs and diluted 100 times in fresh medium. A) Absorbance at 490 nm, and B) fluorescence (Ex. = 490 nm, Em. = 535 nm) were determined at regular intervals (VICTOR *X*3 Multilabel Plate Reader, PerkinElmer). C) Generation number was determined by plating cultures on selective and non-selective LA plates. Data were analyzed with an unpaired two-tailed *t*-test, and significant data are denoted with an asterisk (**: p<0.001; ***: p<0.0001).

The GFPmut3 reporter used in this study showed high resistance to photo bleaching in *P. aeruginosa* (Figure 3A and B). Fluorescence intensity remained stable during the first 5 min of continuous exposure to UV light, although a significant loss of signal was observed after 7 min (p=0.0299). Fluorescent labeling with pUCP18-MCS*gfpmut3* was successfully used to study *P. aeruginosa* ATCC® 10145GFP™ interaction with airway epithelial cells by fluorescence microscopy (Figure 3C).

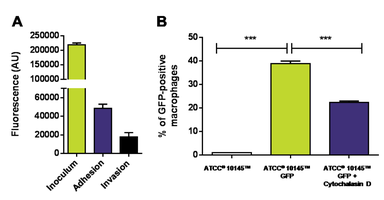
Figure 3: Detection of *P. aeruginosa* ATCC® 10145GFP™ by fluorescence microscopy



A) *P. aeruginosa* ATCC® 10145GFP™ was exposed to UV light (100 W) and imaged at regular intervals (Axioskop™, ZEISS, x100 oil immersion, 505 nm filter). B) Image intensity was measured in 9 independent areas of 200 x 200 pixels using ImageJ (www.rsbweb.nih.gov/ij/), and are represented in arbitrary units. Data were analyzed with an unpaired two-tailed *t*-test and significant data are denoted with an asterisk (*: p<0.01; **: p<0.001; ***: p<0.0001). C) *P. aeruginosa* ATCC® 10145GFP™ interaction with ATCC® CCL-185™ A549 airway epithelial cells (Axioskop, ZEISS, x100 oil immersion). Bacteria appear in green (505 nm), nuclei are stained with DAPI (475 nm, blue), and the cytoskeleton is stained with Alexa Fluor® 555 Phalloidin (565 nm, red) (Invitrogen™).

GFP-labeled *P. aeruginosa* was tested in a high-throughput adhesion and internalization assay in the presence or absence of a phagocytosis inhibitor (Cytochalasin D, Sigma-Aldrich®). Bacterial attachment and internalization by airway epithelial cells (Figure 4A) or murine macrophages (Figure 4B) was detected using a fluorescence microplate reader (Figure 4A) or a flow cytometer (Figure 4B). This proof-of-concept experiment indicates that these fluorescent microorganisms can be used in high-content *in vitro* assays in 96-well plates to screen small molecule inhibitors and study their effect on pathogen-host interactions.

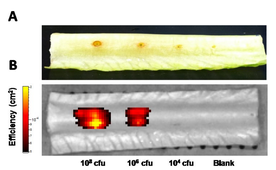
Figure 4: High-throughput detection of pathogen-host interactions



A) ATCC® 10145™ and ATCC® 10145GFP™ were incubated in the presence of ATCC® CCL-185™ A549 airway epithelial cell monolayers in 96-well plates at a MOI of 50. After 1 hour, the cells were washed (Adhesion) and the medium was replaced by F12-K supplemented with 100 µg/mL of gentamicin for 1 hour. Cells were washed again and fluorescence was measured (Invasion) (Ex. = 490 nm, Em. = 535 nm, VICTOR *X*3 Multilabel Plate Reader, Perkin Elmer). B) *P. aeruginosa* ATCC® 10145™ and ATCC® 10145GFP™ were incubated with ATCC® TIB-67™ J774A.1 macrophages at a MOI of 20 in the presence or absence of 50 nM Cytochalasin D (Sigma-Aldrich) and analyzed by flow cytometry using a FACSCalibur™ (BD). Data were analyzed with an unpaired two-tailed *t*-test, and significant data are denoted with an asterisk (***: p<0.0001).

To test the utility of this GFP-reporter for pathogenesis studies, we used an established plant host model system[2] that has been successfully used for high-throughput analysis of virulence and drug discovery. The mid-ribs of *Lactuca sativa* L. var. *longifolia* were infected with various concentrations of ATCC® 10145GFP™, and fluorescence was measured using an *in vivo* imaging system. The assay detected 10^6 to 10^9 cfu/site of infection, indicating that this vector can be successfully used to monitor bacterial growth in the plant host (Figure 5).

Figure 5: *In vivo* detection of *P aeruginosa* ATCC® 10145GFP™



Various concentrations of *P. aeruginosa* ATCC® 10145GFP™ were injected into the mid-rib of *Lactuca sativa* L. var. *longifolia*. A) The area of necrosis caused by *P. aeruginosa* ATCC® 10145GFP™ was measured in mm. B) Fluorescence was detected after 48 hrs using an IVIS® Spectrum (PerkinElmer) detection system.

Conclusions

The *Pseudomonas*-GFP pathogenesis model system presented here clearly demonstrates a proof-of-concept for labeling Gram-negative bacteria for studying pathogenicity. This model system can also be used for screening compounds against *P. aeruginosa* and other important nosocomial pathogens such as *E. coli*, *S. enterica*, or *S. flexneri*.

[1] Choi KH, Schweizer HP. mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. Nat Protoc 1: 153-161, 2006.
[2] Starkey M, Rahme LG. Modeling *Pseudomonas aeruginosa* pathogenesis in plant hosts. Nat Protoc 4: 117-123, 2009.