APPLICATION NOTE

NOVEL FLUORESCENT REPORTERS FOR STUDYING HOST-PATHOGEN INTERACTIONS

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ABSTRACT

We have developed green fluorescent protein (GFP)-labeled strains of various gram-negative opportunistic pathogens from the ATCC collection. These products are compatible with various fluorescence detection technologies such as a fluorescence plate reader, microscopy, flow cytometry, and in vivo imaging systems. The *Pseudomonas*-GFP pathogenesis model system presented in this application note demonstrates the use of these labeled strains in studying bacterial pathogenesis, host-pathogen interactions, and compound screening assays.

INTRODUCTION

GFP, isolated more than 50 years ago from the jellyfish *Aequorea* victoria,[1] has been used extensively in protein function and localization studies, the analysis of promoter activity and regulation, drug discovery research, and non-invasive imaging.[2,3] 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.

ATCC has developed a vector to express GFP in bacteria for use in pathogenesis and host-pathogen studies. A shuttle vector encoding the GFP variant mut3[4] (pUCP18-MCS*gfpmut3*) was generated and successfully transformed into the following ATCC gram-negative opportunistic pathogens (Table 1): *Escherichia coli* (ATCC® No. 25922™), *Salmonella enterica* subsp. *enterica serovar Typhimurium* (ATCC® No. 14028™), *Shigella flexneri* (ATCC® No. 12022™), and *Pseudomonas aeruginosa* (ATCC® No. 10145™ and 15692™). These GFP strains are compatible with various fluorescence detection technologies, including a fluorescence plate reader, microscopy, flow cytometry, and in vivo imaging systems. GFP-labeled *P. aeruginosa* was used as a model to test the characteristics of the vector and the sensitivity of detection using these technologies.

Table 1: ATCC GFP-labeled microorganisms

ATCC® No.	Species	Reporter
25922GFP™	*Escherichia coli*	GFP
14028GFP™	*Salmonella enterica* subsp. *enterica serovar Typhimurium*	GFP
12022GFP™	*Shigella flexneri*	GFP
10145GFP™	*Pseudomonas aeruginosa*	GFP
15692GFP™	*Pseudomonas aeruginosa*	GFP

BACTERIAL DETECTION AND QUANTIFICATION

ATCC GFP-labeled microorganisms can be used for a wide range of applications. The expression of a bright GFP variant (*gfpmut3*) on a high-copy number plasmid facilitates visual identification when exposed to UV-light (Figure 1A) or imaged using a detection system such as the IVIS® Spectrum (PerkinElmer) (Figure 1B and 1C). Quick colony differentiation of GFP-labeled microorganisms from unlabeled organisms or contaminants can also be easily performed by using a hand-held UV wand.

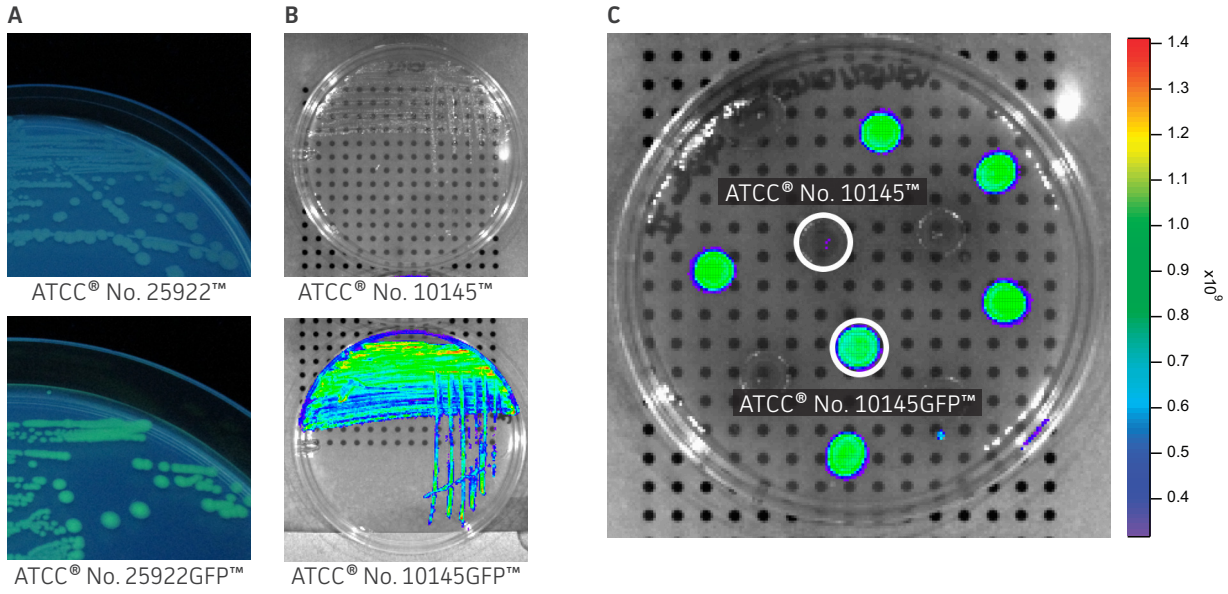


Figure 1: Visual detection of GFP-labeled microorganisms. A) An empty vector control strain (ATCC® No. 25922™) and *E. coli*-GFP (ATCC® No. 25922GFP™) were grown at 37˚C in Lysogeny agar (LA) with 100 µg/mL carbenicillin. Colonies were imaged using a UV Wand (Ultra Violet Products). B) An empty vector control strain (ATCC® No. 10145™) and *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) were streaked on LA with 300 µg/mL carbenicillin, incubated for 24 hours (h) at 37˚C, and imaged using an IVIS Spectrum detection system (Ex. = 500 nm, Em. = 540 nm). C) Mixture of *P. aeruginosa* (ATCC® No. 10145™) and *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) colonies on LA with 300 µg/mL carbenicillin, incubated for 24h at 37˚C, and imaged using an IVIS Spectrum detection system (Ex. = 500 nm, Em. = 540 nm). Key to fluorescence intensity units is shown on the right.

GFP EXPRESSION DOES NOT AFFECT BACTERIAL GROWTH AND FITNESS

To determine whether GFPmut3 expression affects growth fitness and viability of the labeled microorganisms (Table 1), the expression of the *gfpmut3* gene was monitored during growth (Figure 2). GFPmut3 labeled *P. aeruginosa* showed the same growth profiles as the parental strain carrying the empty vector (Figure 2A). Fluorescence was easily detected and quantified using a fluorescence microplate reader (Figure 2B). Background fluorescence of the non-labeled wild-type strain was extremely low. 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 2C). These findings show that GFPmut3 labeling does not affect bacterial growth and can easily be used to detect and quantify bacteria using a fluorescence microplate reader.

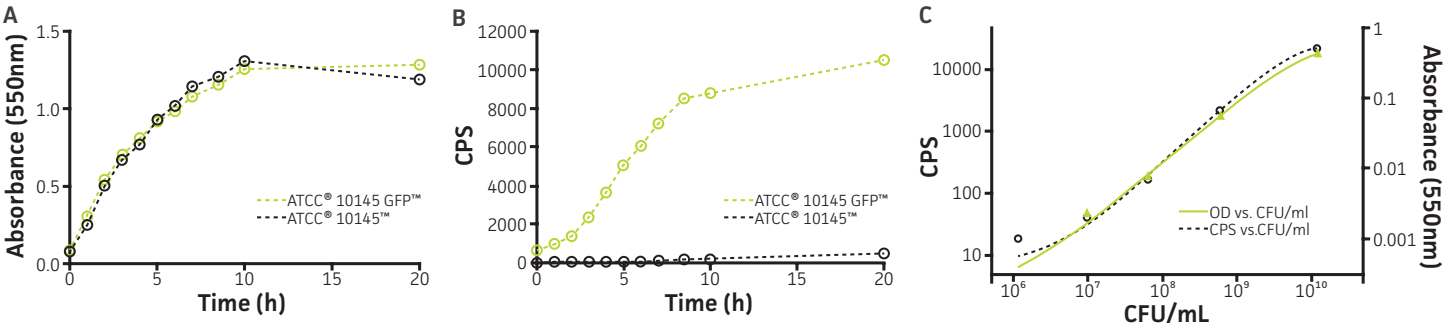


Figure 2: Expression of GFPmut3 does not affect bacterial growth and can be used for bacterial quantification. *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) (green) and an empty vector control (ATCC® No. 10145™) (black) were grown at 37˚C in Lysogeny broth (LB) with 300 µg/mL carbenicillin under constant 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™ X3 Multilabel Plate Reader, PerkinElmer). C) Colony forming units (CFU) were determined by plating appropriate dilutions on LA plates and were compared to CPS (black, R^2=1.000) and absorbance (green, R^2=0.998) using a non-linear regression.

FLUORESCENCE DETECTION AND STABILITY

The stability of the plasmid was tested in *P. aeruginosa*-GFP by passaging a culture in the absence of antibiotic pressure. This was analyzed by measuring fluorescence (Ex. = 490 nm, Em. = 535 nm) every 24h on a microplate reader (Figure 3A and B). The plasmid was stable in *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) for 3 successive passages in the absence of antibiotics and for a minimum of 20 generations (Figure 3C). Together with the long half-life of GFPmut3 in bacteria (over 3 days, data not shown), these results indicate that the fluorescence of GFPmut3 labeled microorganisms is very stable.

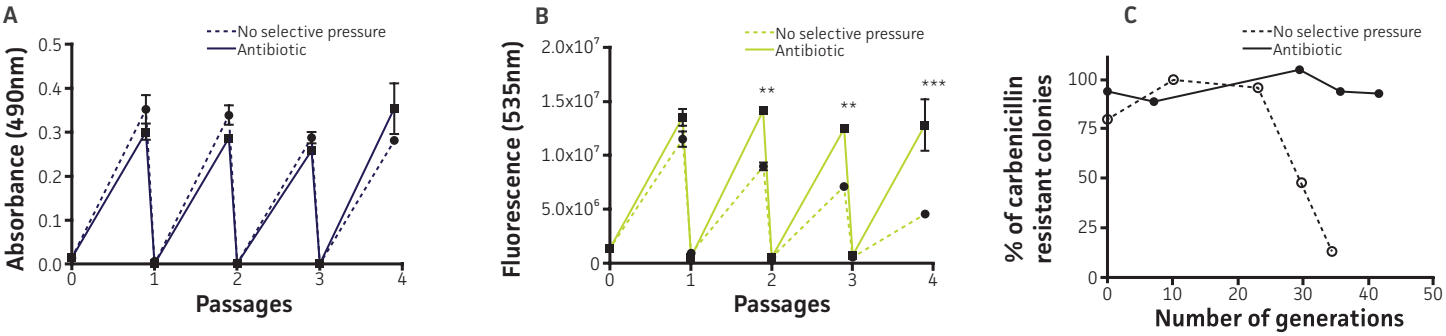


Figure 3: Plasmid stability in *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) was grown at 37˚C in LB with 300 µg/mL carbenicillin (solid line), or in the absence of antibiotic pressure (dashed line), under constant shaking. Cultures were passaged every 24h and diluted 100 times in fresh medium. A) Absorbance at 490 nm, and B) fluorescence (Ex. = 490 nm, Em. = 535 nm) were determined at regular intervals on a microplate reader. 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 (**: $p<0.001$; ***: $p<0.0001$).

DETECTION OF ATCC GFP-LABELED MICROORGANISMS BY FLUORESCENCE MICROSCOPY

The utility of fluorescent proteins is often limited by their sensitivity to photo bleaching. The resistance of *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) to photo bleaching was tested under continuous exposure to UV light. The fluorescence remained stable during the first 5 minutes (m), a significant loss of signal was however observed after 7m of exposure to UV light. The GFPmut3 reporter used in this study therefore shows resistance to photo bleaching in *P. aeruginosa* (Figures 4A and 4B).

To visualize bacterial interaction with eukaryotic cells, *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) was incubated with A549 (ATCC® No. CCL-185™) airway epithelial cells followed by fixation with 2% paraformladehyde and counterstaining of nuclei (blue) and actin (red). The pseudocolored images show that fluorescently labeled *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) can be detected and used for studying interactions with mammalian cells by fluorescence microscopy (Figure 4C).

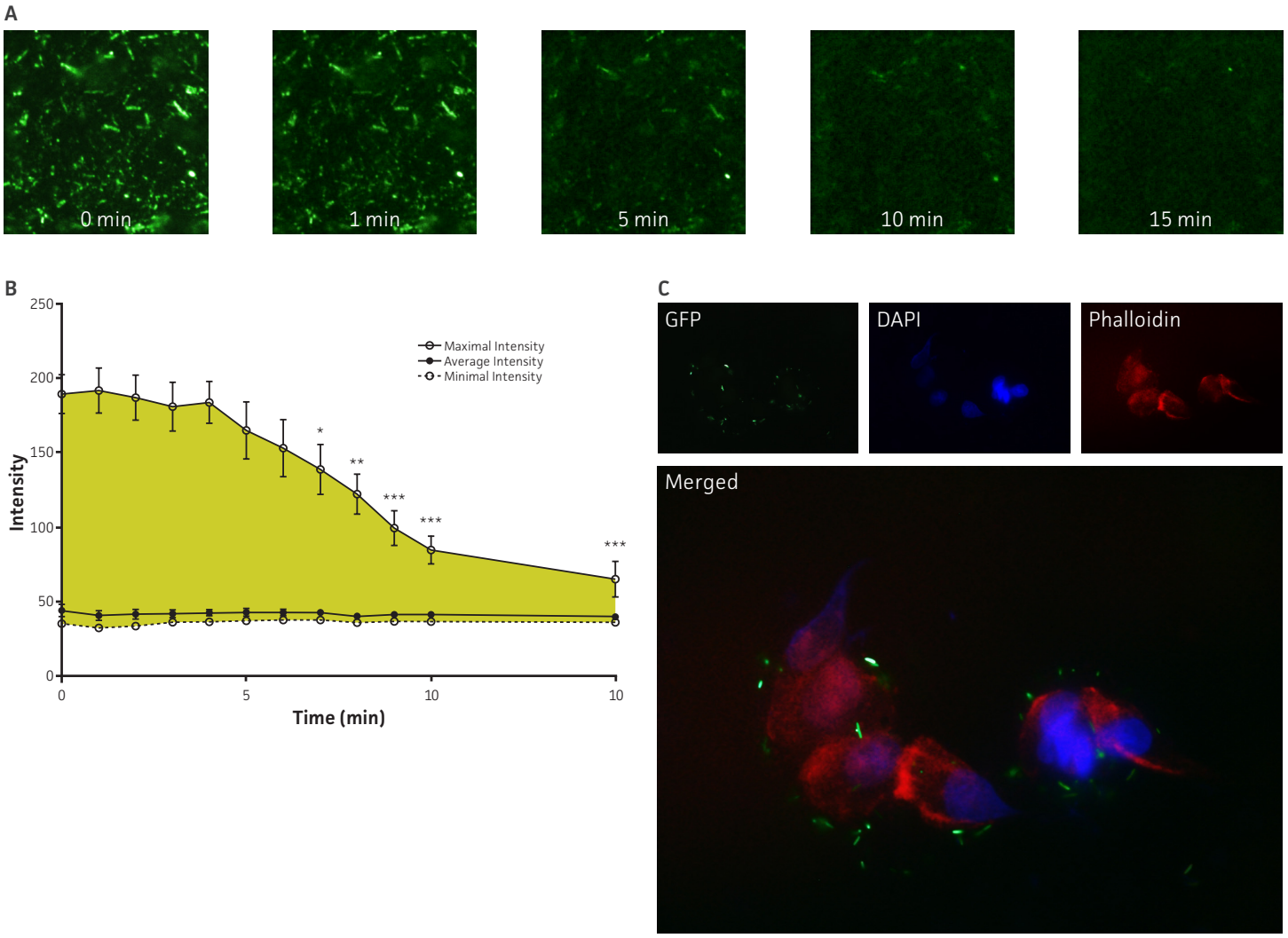


Figure 4: Detection of *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) by fluorescence microscopy. A) *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) was exposed to UV light (100 W) and imaged every 60 seconds (Axioskop™, ZEISS, x100 oil immersion, 505 nm filter). B) Image intensity was measured in 9 independent areas of 200 x 200 pixels using ImageJ (rsbweb.nih.gov/ij/), and is represented in arbitrary units. Data were analyzed with an unpaired two-tailed t-test and data significant from the non exposed control are denoted by an asterisk (*: p<0.01; **: p<0.001; ***: p<0.0001). C) P. *aeruginosa*-GFP (ATCC® No. 10145GFP™) interaction with A549 (ATCC® No. CCL-185™) airway epithelial cells (x100 oil immersion). Bacteria appear in green (505 nm), nuclei were stained with DAPI (475 nm, blue) (Invitrogen™), and the cytoskeleton was stained with Alexa Fluor® 555 Phalloidin (565 nm, red) (Invitrogen).

IN VITRO DETECTION OF HOST-PATHOGEN INTERACTION

P. *aeruginosa*-GFP (ATCC® No. 10145GFP™) was tested in a high-throughput adhesion and internalization assay in 96-well plates using a fluorescence microplate reader and by flow cytometry. The sensitivity of the assay was tested using a phagocytosis inhibitor (Cytochalasin D, Sigma-Aldrich®). Bacterial attachment and internalization by airway epithelial cells was detected using a fluorescence microplate reader (Figure 5A). 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 also to study pathogen-host interactions.

Bacterial fluorescence (Figure 5B), attachment, and internalization by murine macrophages (Figure 5C) were also detected using a flow cytometer. These data show that macrophages with GFP-labeled *Pseudomonas* can be detected using flow cytometry. Overall, GFPmut3 labeled reporters can be used for studying interactions of various pathogens with eukaryotic cells using flow cytometry.

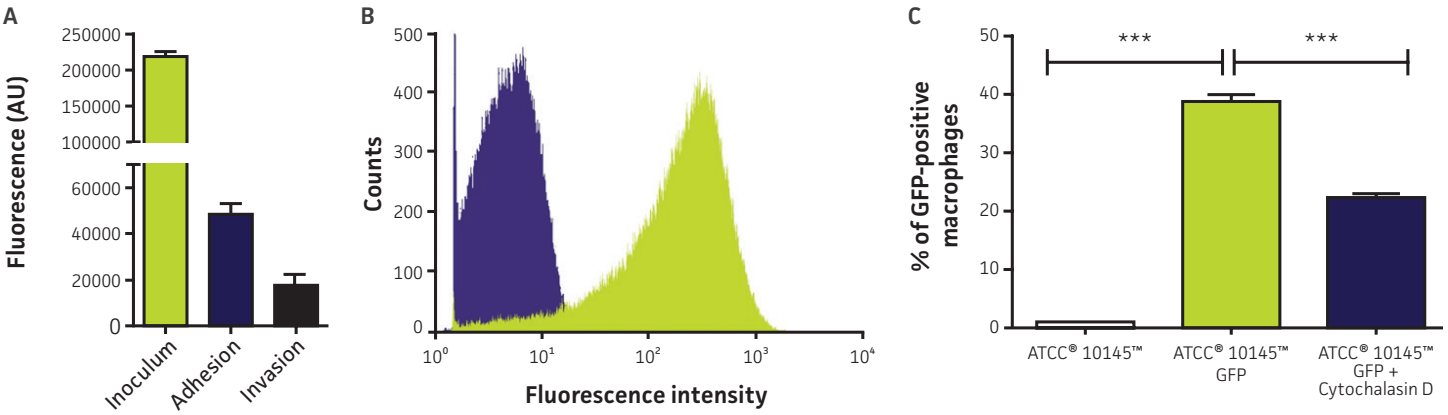
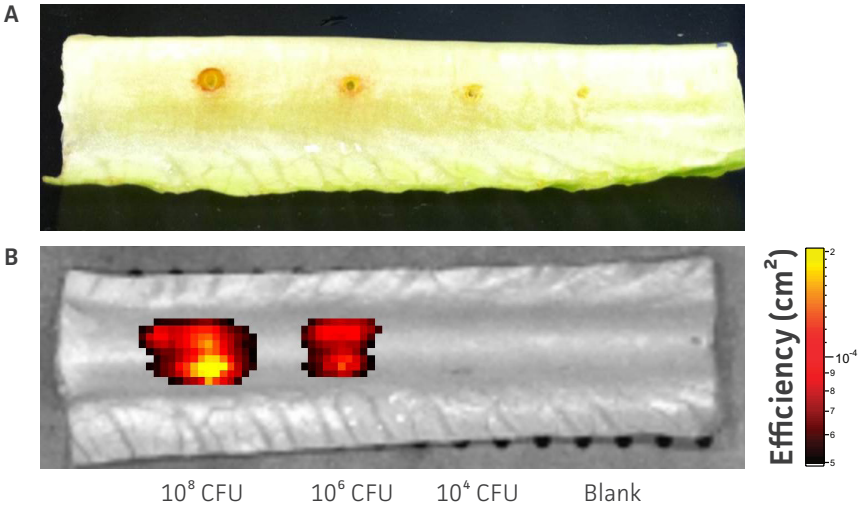


Figure 5: High-throughput detection of pathogen-host interactions. A) *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) was incubated in the presence of A549 (ATCC® No. CCL-185™) airway epithelial cell monolayers in 96-well plates at a multiplicity of infection (MOI) of 50. After 1h, the cells were washed (Adhesion) and the medium was replaced by F12-K supplemented with 100 µg/mL of gentamicin for 1h to kill extracellular bacteria. Cells were washed again and fluorescence was measured in arbitrary units (AU) on a microplate reader (Invasion) (Ex. = 490 nm, Em. = 535 nm). B) *P. aeruginosa* (ATCC® No. 10145™) (purple) and *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) (green) bacterial suspensions were analyzed by flow cytometry using a FACSCalibur™ (BD). C) *P. aeruginosa* (ATCC® No. 10145™) and *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) were incubated with J774A.1 (ATCC® No. TIB-67™) macrophages at a MOI of 20 in the presence or absence of 50 nM Cytochalasin D and analyzed by flow cytometry. Data were analyzed with an unpaired two-tailed t-test, and significant values are denoted with an asterisk (***: $p<0.0001$).

IN VIVO DETECTION OF GFP-LABELED *P. AERUGINOSA*

To test the utility of this GFP-reporter for pathogenesis studies, we used an established plant host model system[5] that has been successfully used for high-throughput analysis of virulence and in drug discovery. The mid-ribs of *Lactuca sativa* L. var. *longifolia* were infected with various concentrations of *P. aeruginosa*-GFP (ATCC® No. 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 employed to monitor bacterial growth in the plant host (Figure 6).

Figure 6: In vivo detection of *P. aeruginosa*-GFP (ATCC® No. 10145GFP™). Various doses of *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) were injected into the mid-rib of *Lactuca sativa* L. var. *longifolia*. A) The area of necrosis caused by *P. aeruginosa*-GFP (ATCC® No. 10145GFP™) was measured in mm. B) Fluorescence was detected after 48h using an IVIS Spectrum detection system (Ex. = 500 nm, Em. = 540 nm).



CONCLUSION

ATCC has developed a vector to express GFP in pathogenic bacteria for use in bacterial pathogenesis and host-pathogen interaction studies. The vector is stable and GFP expression did not alter bacterial growth. The GFP-labeled *P. aeruginosa* (ATCC® No. 10145GFP™) model system represents a proof-of-concept for detecting gram-negative bacteria using a fluorescence plate reader, microscopy, flow cytometry, and in vivo imaging technologies. Similarly, *P. aeruginosa* (ATCC® No. 15692GFP™), *E. coli* (ATCC® No. 25922GFP™), *S. enterica* subsp. *enterica serovar Typhimurium* (ATCC® No. 14028GFP™), and *S. flexneri* (ATCC® No. 12022GFP™) can be used for similar research and compound screening applications using a multitude of detection systems.

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REFERENCES

1 Shimomura, O., Johnson, F. H. & Saiga, Y. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. J Cell Comp Physiol 59, 223-239 (1962).

2 O'Toole, R., Von Hofsten, J., Rosqvist, R., Olsson, P. E. & Wolf-Watz, H. Visualisation of zebrafish infection by GFP-labelled *Vibrio* anguillarum. Microb Pathog 37, 41-46, doi:10.1016/j.micpath.2004.03.001 (2004).

3 Stuurman, N. et al. Use of green fluorescent protein color variants expressed on stable broad-host-range vectors to visualize rhizobia interacting with plants. Mol Plant Microbe Interact: MPMI 13, 1163-1169, doi:10.1094/MPMI.2000.13.11.1163 (2000).

4 Choi, K. H. & Schweizer, H. P. mini-Tn7 insertion in bacteria with single attTn7 sites: example *Pseudomonas aeruginosa*. Nat Protoc 1, 153-161, doi:10.1038/nprot.2006.24 (2006).

5 Starkey, M. & Rahme, L. G. Modeling *Pseudomonas aeruginosa* pathogenesis in plant hosts. Nat Protoc 4, 117-124, doi:10.1038/nprot.2008.224 (2009).

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