Bioengineered 4:1, 50–54; January/February 2013; © 2013 Landes Bioscience

"Light-tagged" bacteriophage as a diagnostic tool for the detection of phytopathogens

David A. Schofield,[1,*] Carolee T. Bull,[2] Isael Rubio,[3] W. Patrick Wechter,[4] Caroline Westwater[5] and Ian J. Molineux[6]

[1]Guild Associates, Inc.; Charleston, SC USA; [2]USDA; Agricultural Research Service; Salinas, CA USA; [3]California State University, Monterey Bay; Undergraduate Research Opportunities Center; Seaside, CA USA; [4]USDA; Agricultural Research Service; U.S. Vegetable Laboratory; Charleston, SC USA; [5]Department of Craniofacial Biology; Medical University of South Carolina; Charleston, SC USA; [6]Molecular Genetics and Microbiology; Institute for Cellular and Molecular Biology; University of Texas at Austin; Austin, TX USA

Detection of the phytopathogen *Pseudomonas cannabina* pv *alisalensis*, the causal agent of bacterial blight of crucifers is essential for managing this disease. A phage-based diagnostic assay was developed that detects and identifies *P. cannabina* pv *alisalensis* from cultures and diseased plant specimens. A recombinant "light-tagged" reporter phage was generated by integrating the *luxAB* genes into the *P. cannabina* pv *alisalensis* phage PBSPCA1 genome. PBSPCA1::*luxAB* is viable, stable and detects *P. cannabina* pv *alisalensis* within minutes and with high sensitivity by conferring a bioluminescent signal. Detection is dependent on cell viability since cells treated with a bactericidal disinfectant are unable to elicit a signal. Importantly, the reporter phage detects *P. cannabina* pv *alisalensis* from diseased plant specimens indicating the potential of the diagnostic for disease identification. The reporter phage displays promise for the rapid and specific diagnostic detection of cultivated isolates, and infected plant specimens.

Keywords: disease, phytopathogen, crucifers, reporter phage, diagnostic, bioluminescence

Submitted: 07/25/12

Revised: 09/07/12

Accepted: 09/10/12

http://dx.doi.org/10.4161/bioe.22159

*Correspondence to: David A. Schofield; Email: dschofield@guildassociates.com

Addendum to: Schofield DA, Bull CT, Rubio I, Wechter WP, Westwater C, Molineux IJ; Addendum to. Development of an engineered bioluminescent reporter phage for detection of bacterial blight of crucifers. Appl Environ Microbiol 2012; 78:3592-8; PMID:22427491; http://dx.doi.org/10.1128/AEM.00252-12

Bacteriophages (phages) specifically infect and lyse their bacterial host and cell lysis has been used for many years as a method for specifically identifying target bacteria.[1-3] Phage typing schemes exist for the majority of bacterial species and are sometimes used to identify specific strains within a species.[4,5] The phage typing process is relatively simple; phage dilutions are spotted onto a bacterial lawn, and if the bacteria are sensitive to the phage, lysis results in an area of clearing. Although robust, the methodology requires the maintenance of a large number of phage stocks and bacterial propagating strains. Because pure bacterial cultures must be employed with phage typing, this methodology is not amenable to complex clinical or environmental samples. To address this limitation, phages are being exploited in a variety of technologies including phage-labeling, phage amplification, and reporter phage.[6,7] The latter approach relies on integrating reporter genes into the phage genome to create a genetically engineered phage capable of emitting a detectable signal (usually colorimetric, fluorescent or bioluminescent). In the absence of a target cell, the reporter phage cannot produce a signal. If the target cell is present, the reporter phage binds to specific receptors on the bacterial cell wall, and infects the cell. Phage and reporter gene expression ensues and the subsequent signal provides a positive identification of the bacterium. The genes encoding the bacterial luciferase (*luxAB*) are commonly used as the reporter of choice as following the addition of an aldehyde substrate, luciferase catalyzes a reaction of which one of the products is "light" (**Fig. 1A and B**). The reporter phage system requires minimal processing of samples and although naturally occurring autobioluminescent environmental or clinical samples can occur, they are extremely rare. Consequently, "light-tagged phage" have been shown useful for detection of a variety of human pathogenic bacteria in vitro,

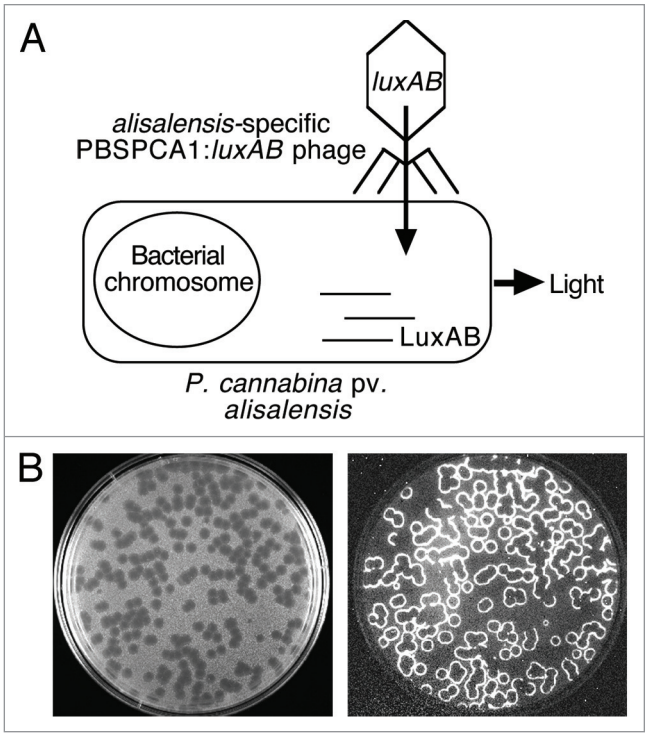

Figure 1. (**A**) Detection overview. The bacterial *luxAB* reporter genes (encoding the luciferase protein) were integrated into the genome of *P. cannabina* pv *alisalensis* phage PBSPCA1 to create a recombinant "*luxAB*-tagged" reporter phage. In the presence of the target cell, the reporter phage binds to a specific receptor and infects the cell. The *luxAB* genes, which are controlled by phage promoters, are expressed using the host's transcriptional and translational machinery to produce the luciferase enzyme; following the addition of the substrate n-decanal and in the presence of oxygen and a flavin mononucleotide, the luciferase catalyzes a complex reaction resulting in light emission. (**B**) "Bioluminescent plaques." A "*luxAB*-tagged" reporter phage was titered on its host strain using soft agar overlays and the plaques were examined under light (left panel) and dark field (right panel) illumination. Note the bioluminescence at the plaque periphery (phage/cell interface) which is indicative of phage-infected cells.

and from complex food and clinical specimens.[8-13] We describe the development of a "*luxAB*-tagged" reporter phage for the detection of an important phytopathogen, and demonstrate that the reporter phage holds promise as a disease diagnostic for bacterial blight.[14]

The phytopathogen *Pseudomonas cannabina* pv *alisalensis* (formerly known as *Pseudomonas syringae* pv *alisalensis*[15]) is the causal agent of bacterial blight, which is a disease afflicting cruciferous vegetables. Cruciferous vegetables are a dominant food crop worldwide and thus a valuable commodity. The disease, which can render the crop unmarketable, is transmissible and there is anecdotal evidence that *P. cannabina* pv *alisalensis* can be a seed contaminant. Phage lysis assays using phage PBSPCA1 (formerly known as PBS1) is used as a standard for the identification of *P. cannabina* pv *alisalensis*.[16-20] PBSPCA1 genome was partially sequenced in order to facilitate molecular engineering of *luxAB* integration. The phage genome is predicted to encode for ~60 proteins, one of which was determined to be a non-essential putative *phoH*-like (phosphate starvation-inducible) protein. This gene was therefore replaced with a *luxAB* gene cassette using homologous recombination. Although most phage can tolerate small size increases in their genome, a replacement strategy was chosen because the resulting recombinant genome would only be ~1.3 kb larger than its parent, and was deemed unlikely to result in the generation of defective reporter phage.

PBSPCA1::*luxAB* phage were isolated following a PCR screening process using successively higher dilutions of phage until single plaques could be picked and propagated. The recombinant genome was confirmed using PCR, and the ability of DNase-1-treated lysates to transduce a bioluminescent signal response to target cells (DNase-1 cannot degrade phage DNA protected by the capsid). The 'fitness' of the reporter phage is comparable to the parental wild-type phage as both exhibited similar lysis curves and lysate titers. PBSPCA1::*luxAB* detects cultured *P. cannabina* pv *alisalensis* within 20 min upon reporter phage addition (**Fig. 2**), indicating that it infects, and produces luciferase rapidly. Incubations for 60 min results in a 10^3-fold increase in signal, but more extended incubations (> 120 min) lead to a gradual signal decline due to cell-mediated lysis. The sensitivity limits of detection are approximately 10^3 CFU/mL, sufficient to detect *P. cannabina* pv *alisalensis* in diseased plant specimens (see **Fig. 4**), but possibly inadequate to reliably detect *P. cannabina* pv *alisalensis* from contaminated seeds. Improvements in sensitivity may be achieved by engineering 2nd generation reporters that maximize the signal. This can be achieved in several ways, including codon optimizing of *luxAB* reporter genes for expression in Pseudomonas and incorporating Pseudomonas transcriptional and translational signals for *luxAB* expression. For example, in a 2nd generation *Yersinia pestis* reporter phage, we integrated *luxAB* downstream of the major capsid gene, resulting in a 10-fold increase in assay sensitivity.[21] Assay sensitivity may also be improved by utilizing phage that are defective in host cell lysis (resulting in greater signal accumulation). Using these or comparable strategies, a detection sensitivity of ~10^2 CFU/mL may be achieved. At very low concentrations (i.e., < 10^2 CFU/mL), the limiting step in detection is unlikely to be the signal strength, but instead the reduced likelihood of phage infection. Low level detection strategies may include collection and concentration of cells prior to phage infection.

In contrast to other detection methodologies, e.g., PCR, hybridization, and immunoanalysis, reporter phage technology will only detect viable cells, while the others will detect live and dead cells. This ability to specifically detect only viable cells is critical if being used in a seed-based

detection assay. For example, if a seed lot was suspected of bacterial infection, then treated by a prescribed seed treatment, the ability to verify if the treatment was effective (i.e., no viable cells remaining) is of considerable importance to the seed company. Reporter phages require the host's transcriptional and translational machinery to express luciferase, and elicit bioluminescence; thus, signal generation is strictly dependent on host metabolic activity (**Fig. 3A**). To demonstrate this attribute, the reporter phage-mediated signal response of metabolically active cells was compared with the response elicited by compromised cells. A *P. cannabina* pv *alisalensis* culture was either left untreated (control) or treated with 70% ethanol for 30 min which resulted in a 10^5-10^6 reduction in viable cell counts (to < 10^2 CFU/mL). As expected, ethanol-treated cells were unable to elicit a bioluminescent signal upon incubation with the reporter phage (**Fig. 3B**). In contrast, for example, PCR detects DNA released from cells into the environment or inside dead cells, potentially resulting in false-positives that lead to unnecessary treatments or loss of seed lots.

We reported previously that the reporter phage detects *P. cannabina* pv *alisalensis* in plants,[14] indicating the ability of this system to be used in a phytopathogen diagnostic setting without the need for extensive and tedious pre-isolation of the bacterium. *Brassica rapa* (turnip greens), when inoculated and grown in a controlled greenhouse environment, developed bacterial blight as indicated by large chlorotic and necrotic areas on leaves. Diseased tissue, when incubated with the reporter phage, elicited a strong bioluminescent signal within 4 h of harvesting. Bacterial blight afflicts many different species of brassicas. *B. rapa*, along with *Brassica juncea* (mustard greens) and *Brassica oleracea* (collards) account for the majority of vegetable brassicas produced in the US. Each of these species is unique and may pose individual challenges in reporter phage diagnostics. For example, *B. juncea* and *B. rapa* have similar leaves, yet *B. juncea* produces large amounts of the thioethers glucosinolate and isothiocyanate. Although small amounts of these compounds can be found in most leafy greens, high amounts may inhibit phage gene expression. Further, the leaf of *B. oleracea* is thick and quite fibrous, features that may interfere with phage infection.

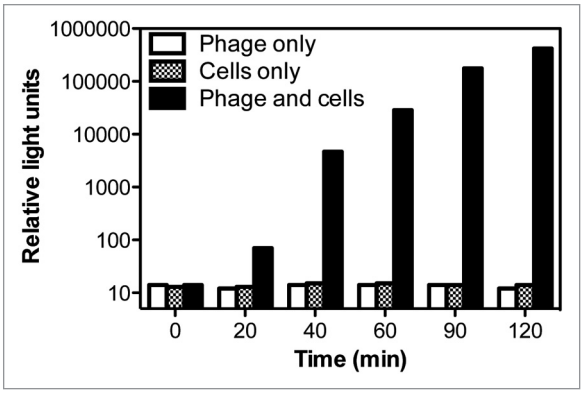

Figure 2. Phage-mediated transduction of a bioluminescent response to *P. cannabina* pv *alisalensis*. Cultures were grown at 28°C in NBY broth until an A_{600} of 0.15–2.0, mixed with the PBSPCA1::*luxAB* reporter phage, and bioluminescence (RLU) was measured over time. Numbers are the mean (n = 3) ± SD.

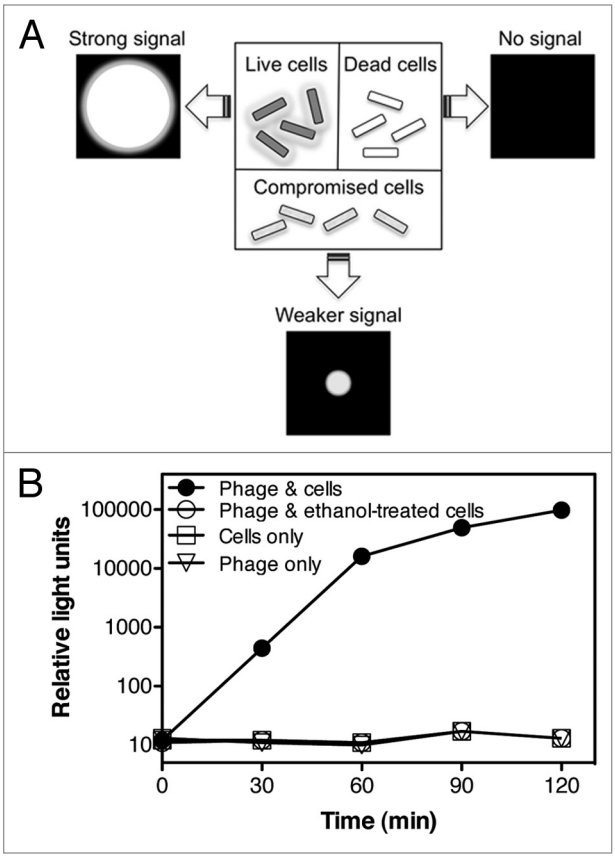

Figure 3. Viable cells are required for a bioluminescent signal response. (**A**) Viable, metabolically active cells are required for phage infection and reporter gene expression. Thus, metabolically active cells should elicit strong signal responses while dead cells will not elicit a signal response. (**B**) A *P. cannabina* pv *alisalensis* culture was divided equally and either left untreated, or incubated with 70% ethanol for 30 min. Following removal of the ethanol, control and treated cells were incubated with the reporter phage and bioluminescence (RLU) was measured over time. Numbers are the mean (n = 3) ± SD.

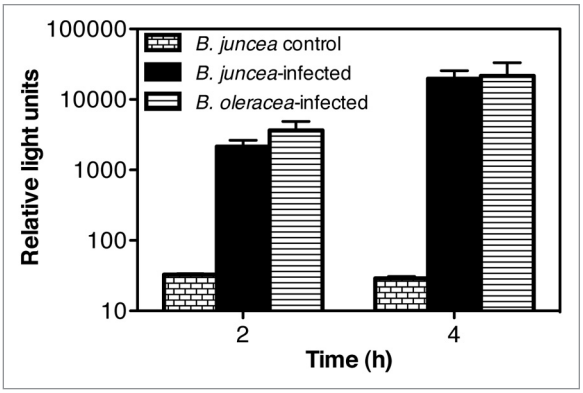

Figure 4. Reporter phage detection of *P. cannabina* pv *alisalensis* from infected *Brassica*. *B. juncea* were grown in the field at the US Vegetable laboratory (Charleston, SC), inoculated with *P. cannabina* pv *alisalensis*, and incubated for 14 d before the infected leaves were harvested. Commercially grown *B. oleracea*, showing symptoms of blight, were sent from a South Carolina grower to the US vegetable laboratory in Charleston for disease identification. An infection resistant cell line of *B. juncea*, grown under the same conditions, was used as a control. One cm discs of control and infected leaf parts were mixed with media, and incubated at 28°C to allow for bacterial elution and outgrowth. At various time points after outgrowth (2 or 4 h), 900 μL of the leaf eluate was placed in a fresh tube, and the reporter phage was added. Bioluminescence was measured 120 min after the addition of the reporter phage. Numbers represent the mean ± SE of 3 independent leaf samples.

We tested whether PBSPCA1::*luxAB* can detect *P. cannabina* pv *alisalensis* in infected *B. juncea* and *B. oleracea*. *P. cannabina* pv *alisalensis*-infected *B. juncea* samples were obtained through experimental inoculation, while *B. oleracea* samples exhibiting characteristics of bacterial blight were provided by a South Carolina commercial grower.

B. juncea was grown in the field at the US Vegetable Laboratory in Charleston, South Carolina and then inoculated with *P. cannabina* pv *alisalensis*. Plants were then maintained in the field under normal weather conditions for 2 weeks before leaves, which exhibited characteristic signs of infection, were harvested and then analyzed with the reporter phage. As a control, a *B. juncea* breeding line, which exhibits resistance to *P. cannabina* pv *alisalensis,* was grown and inoculated under analogous conditions. These plants did not display signs of infection, and as expected, these control tissues did not elicit a bioluminescent signal response with the reporter phage (**Fig. 4**). In contrast, a bioluminescent signal response was evident from the infected leaves following a 2 or 4 h elution and outgrowth period.

B. oleracea leaves showing symptoms of bacterial blight, were sent from the commercial grower to the US Vegetable Laboratory in Charleston, SC for disease identification. In this particular case, over 60% of the grower's 200 acre crop exhibited disease symptoms. Bacteria were isolated from the infected leaf samples using standard culturing methods. The bacteria displayed a blue fluorescent phenotype under UV exposure when grown in King's B medium, a characteristic of *P. cannabina*. Analysis of DNA fragment banding patterns using published BOX-PCR protocols identified the isolate as *P. cannabina* pv *alisalensis*.[22] These same infected leaves were then tested directly with the reporter phage for a bioluminescent signal response. These samples elicited a bioluminescent response as expected (**Fig. 4**). Collectively, this data indicates the ability of the reporter phage to detect *P. cannabina* pv *alisalensis* from different brassicas whether they were artificially infected in the greenhouse, or under field conditions. In addition, the reporter phage was able to detect *P. cannabina* pv *alisalensis* from "authentic" diseased specimens provided by a commercial grower.

Our development of PBSPCA1::*luxAB* represents the first bioluminescent reporter phage assay for an important plant pathogen. The assay functions well with both cultured isolates and naturally diseased specimens. Although there are a number of phage-based diagnostic assays that have been developed and marketed for the detection of human pathogens, it is surprising that phage-based diagnostic assays have not yet been developed within the agricultural industry. In contrast, there are a number of phage diagnostic assays that have been developed and marketed for the detection of human pathogens. For example, phage ϕA1122 is used for confirming identification of *Y. pestis*,[23,24] phage γ is FDA-approved as a standard for the identification of *Bacillus anthracis*,[25] *FASTPlaque*TB™ is used for the detection of *Mycobacterium tuberculosis,* and a cocktail of phages (KeyPath™) are FDA-cleared for the detection of methicillin-sensitive, or -resistant *Staphylococcus aureus*. Although none of these assays utilize genetically engineered reporter phage, the use and implementation of phage diagnostic assays are more developed for the clinical setting. A limiting factor in developing a diagnostic test is the time required for the isolation and selection of a phage that displays the desired host-range, i.e., a phage that displays species specificity and broad-strain infectivity. However, phages are the most abundant entities on earth with estimates ranging from 10^{30} to 10^{32} (ref. 26). Although it is likely that species-specific phages exist that may be used to target any bacterial pathogen of choice, identifying a candidate diagnostic phage can be a time consuming, laborious, and sometimes futile endeavor. Nevertheless, the potential of using phages as diagnostic tools, as in vitro biocontrol agents, or as therapeutic agents in vivo, will undoubtedly result in the development of more and more applications that utilize phage.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This research was supported by the National Science Foundation Small Business Innovative Research grant 1012059 and in part by the US Department of Education and the US Department of Agriculture. Any opinions, findings, conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the funding agencies.

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