Phage Reporter Biosensing of Pathogenic Agents

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ABSTRACT

Considered the most abundant organism on Earth, at a population approaching 10^{31}, bacteriophage mediate interactions with myriad bacterial hosts that has for decades been exploited in phage typing schemes for signature identification of clinical, foodborne, and waterborne pathogens. More recently, this basic premise of phage/host specificity has been advanced via phage-mediated signaling elements that indicate when a phage/host infection event has occurred, thereby providing a very simple and rapid means for bacterial recognition. We have, for example, linked the bacterial bioluminescent (*lux*) response to phage/host infection using quorum sensing chemical synthesis as a signaling intermediary. This permits facile light-based sensing of *E. coli* O157:H7 at concentrations as low as 1 cfu/ml. With the concurrent ability to nanointerface reporter assays onto integrated circuit microluminometers, the potential exists for the development of phage-based sentinel microchips that can function as environmental monitors for multiplexed recognition of biological agents in air, food, and water.

Keywords: bacteriophage, bioluminescence, *Escherichia coli*, *lux*, pathogen

1 INTRODUCTION

Luciferase is a generic name for an enzyme that catalyzes a light-emitting reaction. In bacteria, the genes responsible for this light reaction, referred to as *lux*, have been isolated from organisms such as *Vibrio fischeri* and *Photorhabdus luminescens* and used extensively in the construction of bioluminescent bioreporters that emit blue-green light after contact with a desired chemical target. Target specificity is user controllable via selection of appropriate *lux* linked genes that are activated upon exposure of the living cell to the target. Thus, bioluminescent bioreporters for various chemicals such as polyaromatic hydrocarbons, heavy metals, pesticides, antibiotics, etc. have been produced and tested in solid, liquid, and gaseous sample matrices (see [2] for a review).

The *lux* genes themselves consist of a cassette of five genes, referred to as *luxC*, *luxD*, *luxA*, *luxB*, and *luxE*, (or more simply shortened to *luxCDABE*) whose products are necessary for the generation of light, and two genes (*luxI* and *luxR*) that produce the components of a quorum sensing system that activates transcription of the *luxCDABE* cassette [4]. The luciferase genes (*luxAB*) encode the proteins responsible for generating bioluminescence, while the reductase (*luxC*), transferase (*luxD*), and synthetase (*luxE*) genes encode for proteins that produce an aldehyde substrate required for the bioluminescent reaction. With all genes present, the cell itself drives the bioluminescent reaction; no substrate additions or other user interactions are necessary, thus making the bioreporter a self-directed and self-sustaining sensing and monitoring device.

In addition to chemical sensing, a variant of bioreporter sensing has additionally been designed to detect biological pathogens. These sensors use bacteriophage (bacterial viruses), or phage for short, to identify the pathogen of interest. Phage can be highly specific as to which pathogen they attach to and infect, and by choosing appropriate phage or cocktails of several phage, one can uniquely target a desired bacterium down to the species or even strain level. The *luxA* and *luxB* genes were initially inserted into phage genomes, and upon host cell infection were transferred into and became part of the bacterial host. The subsequent expression of *luxAB* by the bacterial host in tandem with user addition of an aldehyde substrate (required since the *luxCDE* genes are not present) led to bioluminescence endpoint signaling of pathogen presence. Microbes such as *Listeria* and *E. coli* O157:H7 could be detected at less than one pathogen per gram of contaminated food under pre-enrichment conditions [3].

To bypass aldehyde substrate addition and evolve towards an autonomous pathogen sensing system, we paired *luxCDABE* bioreporters with *lux* incorporated phage to create phage reporters that, upon host pathogen infection, liberate synthesis of a chemical target that is then specifically detectable by a bioluminescent bioreporter [1, 5]. To do so, the *V. fischeri luxI* gene, responsible for the synthesis of the acylhomoserine lactone (AHL) autoinducer chemical *N*-3-(oxohexanoyl)-L-homoserine lactone (OHHL), was incorporated into the genome of the *E. coli* O157:H7 specific phage PPO1. Upon infection of *E. coli* O157:H7 by this $PPO1_{luxI}$ reporter phage, the *luxI* gene is expressed and the *E. coli* cells synthesize and release OHHL. A bioluminescent bioreporter tuned to the detection of OHHL was additionally created to complete the sensory circuit. The assay functions by first adding the $PPO1_{luxI}$ reporter phage to the sample. If *E. coli* O157:H7 cells are present, infection occurs, OHHL is synthesized and subsequently detected by the bioluminescent bioreporter, which then emits the light signal. The circuitous route from detection to bioluminescent signaling is illustrated in Figure 1.

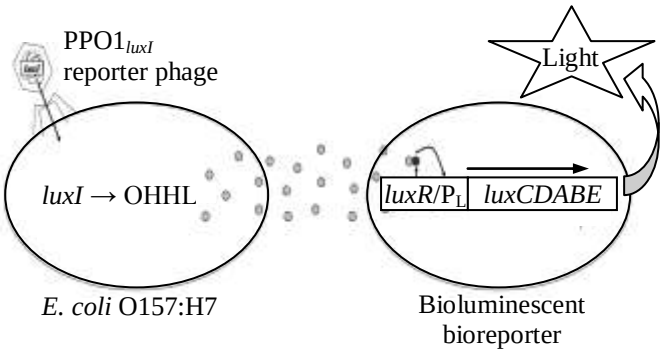

Figure 1: The interplay of pathogen specific phage reporters ($PPO1_{luxI}$) and chemical specific bioluminescent bioreporters yields an autonomous light generating assay

2 APPLICATIONS OF THE PHAGE REPORTER ASSAY

2.1 Detecting *E. coli* O157:H7 in pure culture

To determine the limits of the phage reporter assay under ideal conditions, a dilution series of *E. coli* O157:H7 (ATCC strain 43888) ranging from 1×10^7 cfu/ml to 1 cfu/ml was prepared in pure culture. In a 96 well microtiter plate, 100 µl of each *E. coli* O157:H7 dilution was combined with 100 µl of $PPO1_{luxI}$ reporter phage (~1×10^9 pfu/ml final concentration) and 50 µl of the OHHL specific bioluminescent bioreporter cells (~5×10^6 cfu/ml final concentration), referred to as bioreporter OHHLux. The microtiter plate was then placed in a BioTek Synergy2 Microplate reader (Winooski, VT) programmed to collect bioluminescence every 20 min at an integration time of 1 sec/well with 27ºC incubation. At the highest concentration of 1×10^7 cfu/ml, *E. coli* O157:H7 could be detected within 2 hours (Figure 2). The lowest directly detectable concentration was 1×10^3 cfu/ml, which occurred within 5 hours. Concentrations below 10^3 cfu/ml could not be detected without an additional 6 hour pre-incubation step. This additional 6 hours of pre-incubation then permitted detection of 100 cfu/ml within 1 hour (~7 hours total assay time), 10 cfu/ml within 2.5 hours (~8.5 hours total), and 1 cfu/ml within 4.5 hours (~10.5 hours total). An additional 0.5 hours can be added to the total assay times to account for culture preparations and loading of microtiter plates. Once plate preparation is complete and placed in the reader instrument, however, no other user interaction is required except for data analysis at the termination of the assay.

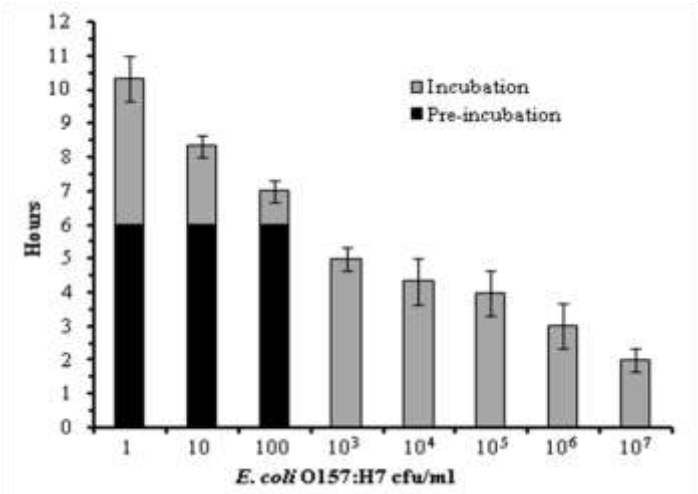

Figure 2: Response time profile of bioluminescence emission in a pure culture 1:10 dilution series of *E. coli* O157:H7 (n = 3)

2.2 Detecting *E. coli* O157:H7 in apple juice

Store purchased pasteurized apple juice was artificially contaminated in 25 or 50 ml volumes with *E. coli* O157:H7 in a 1:10 dilution series down to 1 cfu/ml, and then stored overnight at 4ºC. The next day, dilution tubes were centrifuged and pellets resuspended in 100 µl fresh apple juice and transferred to a 96 well microtiter plate. $PPO1_{luxI}$ reporter phage and OHHLux bioluminescent bioreporters were then added as described for the pure culture experiments, and assayed in the BioTek Synergy2 instrument. Without pre-incubation, detection from 1×10^5 to 1×10^7 cfu/ml occurred within less than 5 hours (Figure 3). At lower concentrations, a 6 hour pre-incubation step was required to elevate *E. coli* numbers prior to assay initiation. This then permitted detection down to 100 cfu/ml. At concentrations of 1 and 10 cfu/ml, the initial apple juice inoculum volume needed to be increased from 25 ml to 50 ml, which then permitted detection within less than 24 hours inclusive of a 6 hour pre-incubation.

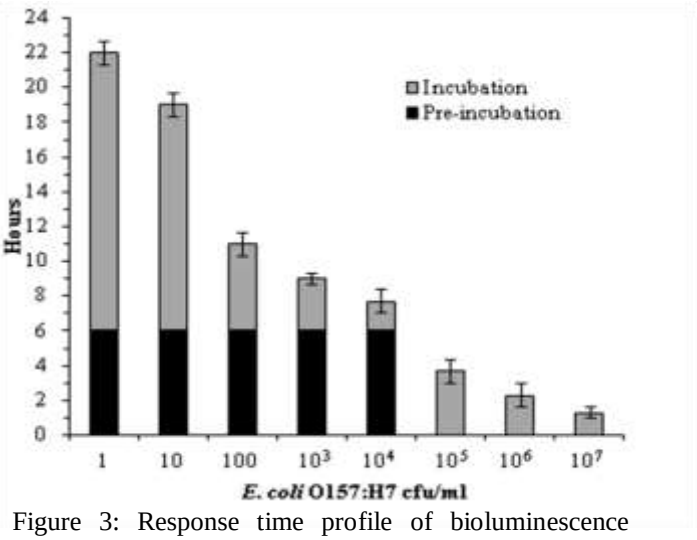

Figure 3: Response time profile of bioluminescence emission in 1:10 dilution series of *E. coli* O157:H7 prepared in apple juice (n = 3)

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2.3 Detecting *E. coli* O157:H7 in tap water

A 1:10 dilution series of *E. coli* O157:H7 was prepared in 25 ml volumes of unadulterated tap water, stored overnight at 4ºC, and then centrifuged the next day with resulting pellets resuspended in 100 µl fresh tap water. After transfer to 96 well microtiter plates, PPO1$_{luxI}$ reporter phage and OHHLux bioluminescent bioreporters were added as described for the pure culture experiments. The 1 × 10^7 to 1 × 10^5 cfu/ml cultures were detectable without pre-incubation within 5 to 7 hours, respectively (Figure 4). Lower concentrations required a 6 hour pre-incubation, permitting detection within times ranging from 8.5 hours at 1 × 10^4 cfu/ml up to 12.5 hours at 1 cfu/ml.

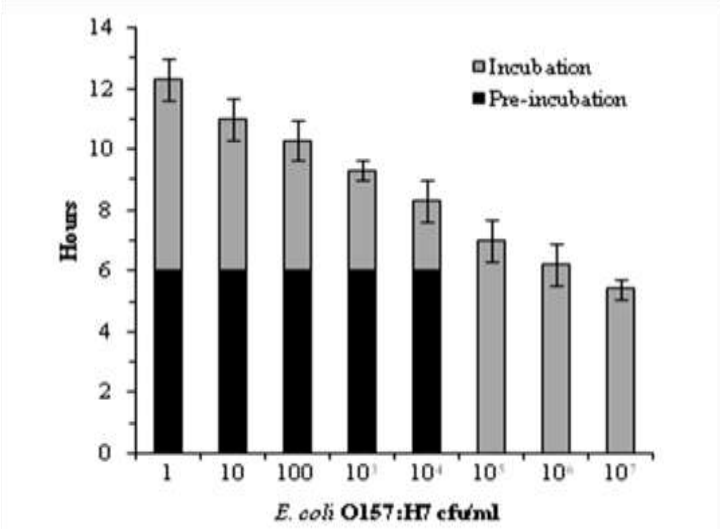

Figure 4: Response time profile of bioluminescence emission in 25 ml sample volumes of *E. coli* O157:H7 contaminated tap water (n = 3)

2.4 Real-time bioluminescent imaging of *E. coli* O157:H7 in spinach leaf rinsate

Since bioluminescence can be measured using any variety of CCD imaging cameras, such as the IVIS Lumina from Caliper Life Sciences (Alameda, CA), the opportunity to monitor contamination events in real-time becomes feasible. We attempted real-time imaging using rinsate from artificially contaminated spinach. Spinach leaves were immersed in *E. coli* O157:H7 contaminated water dilutions ranging from 1 × 10^7 to 1 cfu/ml, left overnight at 4ºC, and the next day transferred to sterile petri dishes and placed in a sterile fume hood for 30 minutes to promote drying. Contaminated leaves were then transferred into 20 ml volumes of Luria-Bertani (LB) media and incubated for 2 hours at 40ºC. The entire 20 ml volumes were then individually placed in stomacher bags and stomached for 2 minutes. Each liquid portion was removed, centrifuged for 10 minutes at 6000 rpm, and the resulting pellets resuspended in 1.5 ml of LB. Anti-*E. coli* O157:H7 immunomagnetic beads (20 µl) (Dynal Dynabeads, Invitrogen, Carlsbad, CA) were added, gently mixed for 10 minutes at room temperature, and magnetized for 3 min. Captured magnetic bead/*E. coli* complexes were resuspended in 1 ml LB and transferred to 35 mm petri dishes to which was added 1 ml of PP01$_{luxI}$ reporter phage and 0.5 ml of OHHLux bioluminescent bioreporters. Petri dishes were placed in the light-tight IVIS Lumina imaging chamber set at 27ºC and monitored for bioluminescence emission every 5 minutes at an integration time of 1 minute to produce a time series of images of bioluminescence emanating from each *E. coli* contaminated dish. Since pre-incubations were performed, final *E. coli* concentrations did not follow the original 1:10 dilution series, as noted in Figure 5. At the highest *E. coli* O157:H7 concentration of 3 × 10^9 cfu/ml, real-time imaging confirmed detection within 1.5 hours after the 2 hour pre-incubation. At the lowest *E. coli* O157:H7 concentration of 1 × 10^3 cfu/ml (derived from the original inoculum of 1 cfu/ml), detection occurred within approximately 4 hours after the 2 hour pre-incubation.

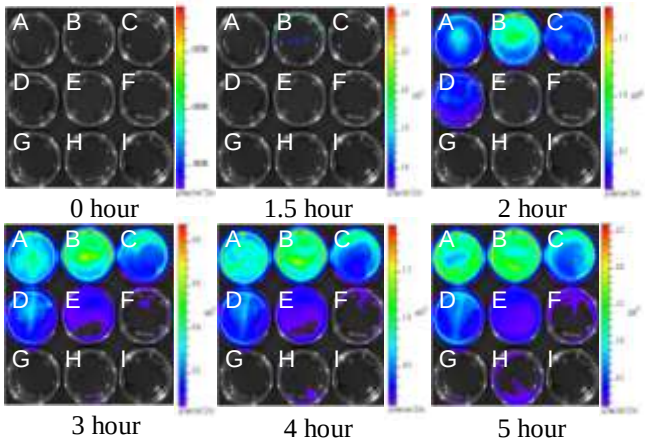

Figure 5: Real-time bioluminescent imaging of *E. coli* O157:H7 contaminated spinach leaf rinsates in nine 35 mm petri dishes over a 5 hour period. Final concentrations of *E. coli* in each petri dish, as determined by viable counts, were A, 5.6 × 10^8; B, 2.7 × 10^9; C, 6.9 × 10^7; D, 4.2 × 10^6; E, 8.8 × 10^5; F, 1.5 × 10^4; G, 1.3 × 10^3; and H, 2.3 × 10^4 cfu/ml. The petri dish labeled 'I' served as a control void of an *E. coli* O157:H7 inoculum.

3 DISCUSSION

The natural specificity of a phage for its particular bacterial host provides a unique assaying mechanism for pathogen monitoring and detection. Since phage infect only living cells, their use ensures detection of viable target, as opposed to immunological (ELISA) or nucleic acid (PCR)-based assays that can falsely signal in response to cellular remnants or free nucleic acid. Further, the ease of manufacturing large quantities of phage and their long

shelf-life and associated low cost further merits their practical application for biological sensing. In these studies, it has been shown that phage reporter assays based on the bioluminescent *lux* response can detect *E. coli* O157:H7 down to 1 cfu/ml within less than 24 hours in apple juice and tap water samples. Assay set-up requires minimal user training and a time commitment of less than one hour, and the use of standard 96-well microtiter plates efficiently integrates with common high-throughput testing and robotic workstation formats. With *lux*-based bioluminescence being a fully autonomous response, it was also feasible applying the assay in association with bioluminescent imaging technologies to establish real-time response profiles of *E. coli* O157:H7 contamination on spinach leaves, with detection of original 1 cfu/ml inoculums occurring within 4 hours following a 2 hour pre-incubation.

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