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Note

Characterization and validation of a bioluminescent bioreporter for the direct detection of *Escherichia coli*

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ABSTRACT

A two-component bacteriophage-based bioluminescent reporter system was developed for the detection of *Escherichia coli* in environmental samples. The bioreporter system consists of a *luxI* integrated lambda bacteriophage and a *lux*-based bioluminescent reporter cell that responds to the infection event through acyl-homoserine lactone (AHL) mediated quorum sensing and bioluminescent signal stimulation. This work addresses the ability of the bioreporter system to detect and quantify the target pathogen in response to two analytical challenges: (1) detection of target cells in the presence of lactonase-producing non-target organisms that could interrupt AHL signal transduction, and (2) detection of sub-lethally injured or physiologically stressed target cells. The bioreporter system was able to autonomously respond to lambda phage infection events with a target host *E. coli* at 1×10^8 cfu/mL against a background of lactonase-producing *Arthrobacter globiformis* at cell densities ranging from 1 to 1×10^8 cfu/mL. *E. coli* target cells stressed by carbon-limitation for 2 weeks (i.e., starvation) or exposure to iodine for 1 week at 2 and 20 ppm (i.e., disinfection) yield a reduced, but detectable, biosensor response. Conversely, short-term iodine exposure produces a significant increase in bioreporter response within the first 24 h. The signal response and limit of detection for the two-component bioreporter system were affected by the physiology and environment of the target, but the bioreporter maintained target specificity demonstrating its potential application for remote sensing of pathogens.

The exploitation of phage specificity for their bacterial host has potential for monitoring opportunistic pathogens in many types of environmental samples. Various bacteriophage-based approaches have been employed to detect diverse bacteria and chemical toxins (Casavant et al., 2003; Chen and Griffiths, 1996; Favrin et al., 2001; Funatsu et al., 2002; Goodridge et al., 1999; Goodridge and Griffiths, 2002; Kodikara et al., 1991; Loessner et al., 1996; Mosier-Boss et al., 2003; Oda et al., 2004; Pagotto et al., 1996; Sarkis et al., 1995; Stone, 2002; Ulitzer and Kuhn, 1987; Waddell and Poppe, 2000; Wolber and Green, 1990). In the past, phage assay systems required the addition of a substrate or the use of specialized monitoring equipment that reduced their utility and application relative to other detection methods (Roelof van der Meer et al., 2004). In an effort to reduce the time and reagents necessary; the system tested here is a two-component, recombinant phage system for the detection of *Escherichia coli* that links phage infection events to quorum sensing signal molecule biosynthesis and luminescent bioreporter induction.

A lambda phage was constructed with a *luxI* gene insert (λ_{luxI}) to produce an *E. coli* specific reporter that actively replicates autoinducer (Ripp et al., 2006). The *Vibrio fischeri* specific autoinducer acyl-homoserine lactone (AHL), *N*-3-(oxohexanoyl)-L-homoserine lactone (OHHL), encoded by the *luxI* insert is detected by a specific luminescent bioreporter, OHHLux, that contains the *luxR* and *luxCDABE* genes (Ripp et al., 2006). The interaction of autoinducer with LuxR stimulates *luxCDABE* expression and the bioreporter generates a light signal at a wavelength of 490 nm (Meighen, 1994). Thus, the initial phage infection event yields an autoamplified chemical signature that is sensed and communicated through luminescent bioreporter signal induction (Whitehead et al., 2001).

In the standard assay, fixed volumes of *E. coli* K12 variant XL1-Blue (Stratagene, La Jolla, CA) (100 μL, serial dilutions ranging from ~1×10^8 down to 1 cfu/mL), OHHLux bioreporter cells (50 μL, ~1×10^6 cfu/mL), and λ_{luxI} bacteriophage (100 μL, ~1×10^9 pfu/mL) were dispensed in triplicate into wells in a 96-well microtiter plate (Isoplate, Perkin-Elmer, Wellesley, MA) (Ripp et al., 2006). Microtiter plates were sealed with Breathe-Easy strips (Diversified Biotech, Boston, MA) and loaded into a Victor^2 Multilabel Counter (Perkin-Elmer) for measurement of luminescence at 490 nm. Assays were typically carried out over a 24 h period at 30 °C with 10 min orbital shaking between serial readings. Luminescence signal data was normalized by division of the background control (the average of triplicate wells containing λ_{luxI} phage, OHHLux bioreporter, and LBS diluent void of target *E. coli* XL1-Blue

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cells). Positive responses were scored as luminescence values (NRLUs) that were greater than or equal to two standard deviations (2σ) above the background controls. Controls were included in each run, incorporating all assay components singly as well as in all possible combinations (i.e., *E. coli* XL1-Blue+OHHLux, *E. coli* XL1-Blue+λ_{luxI} phage, etc.).

Luria–Bertani broth plus supplements (LBS) (10 g Bacto tryptone (Difco, San Jose, CA), 5 g Bacto yeast extract (Difco), 5 g NaCl (Sigma-Aldrich, St. Louis, MO), 1 mL 1 M $MgCl_2$ (Sigma), 1.1 mL 1 N NaOH (Sigma), 1 L water) was used for bacterial culture growth. The target organism, *E. coli* XL1-Blue, was cultured in LBS at 30 °C to an optical density at 600 nm (OD_{600}) of 0.6 (~1×10^8 cfu/mL). *E. coli* OHHLux served as the *lux*-based bioreporter strain for the assay and is resistant to phage lambda and the antibiotic kanamycin. The OHHLux bioreporter was grown in LBS with kanamycin (50 μg/mL (Kn_{50})) (Sigma) at 30 °C to an OD_{600} of 0.4 (~1×10^6 cfu/mL). For enumeration, each of the bacterial cultures were serially diluted in sterile deionized water and plated in triplicate on LBS agar (OHHLux on LBS with Kn_{50}) and were incubated at 37 °C overnight.

Phage were propagated and enumerated in Top agar (1 g Bacto tryptone (Difco), 0.5 g Bacto yeast extract (Difco), 0.5 g NaCl (Sigma), 0.6 g agarose (Difco), 1 mL 1 M $MgSO_4$ (Sigma), 1 L water) and stored in SM buffer (50 mM Tris–HCl pH 7.5 (Fisher Scientific, Pittsburgh, PA), 100 mM NaCl (Sigma), 8 mM $MgSO_4$ (Sigma), 0.01% gelatin (Sigma), 1 L water). Phage λ_{luxI} (100 μL) and 100 μL of XL1-Blue from an overnight culture were incubated for 15 min at ambient temperature and for an additional 15 min at 37 °C. This mixture was then added to 3 mL of Top agar cooled to 45 °C and poured over LBS agar plates. Plates were incubated overnight at 37 °C. Plaques were recovered from the plates with SM buffer and the resulting lysate was treated with one drop of chloroform and centrifuged for 10 min at 3000 ×g. For enumeration,

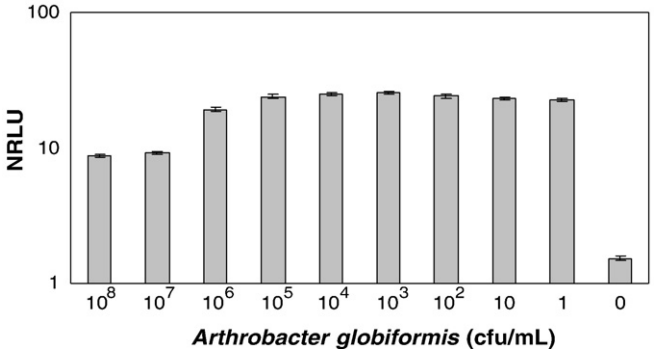

Fig. 2. Mixed culture maximum response peak level. *E. coli* biosensor response levels in Normalized Relative Luminescence Units (NRLU) in mixed culture with *A. globiformis*, a lactonase-producing strain. The concentration of λ_{luxI} bacteriophage was kept constant at 1×10^9 pfu/mL, the concentration of the target host organism XL1-Blue was fixed at 1×10^8 cfu/mL, and the concentration of the bioreporter organism OHHLux was fixed at 1×10^6 cfu/mL while the concentration of challenge *A. globiformis* was manipulated from 1×10^8 to 1 cfu/mL. All concentrations of target host organism XL1-Blue in mixed culture were detected at greater than 2 standard deviations (2σ) above the negative control. The background control had an average maximum peak value of 1.53 NRLU.

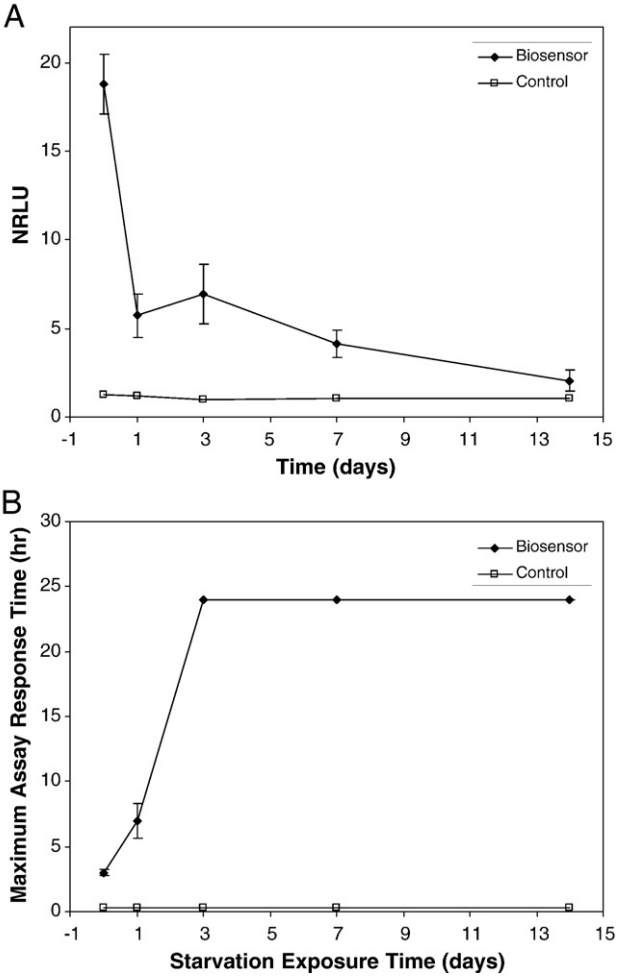

Fig. 1. Effects of starvation on the biosensor. The *E. coli* biosensor assay was better able to detect subtle shifts resulting from environmental stress than those from physiological testing. Within 1 day the bioreporter system was registering a significant decrease in maximum peak height (A) and within 3 days was able to detect a significant increase in the time to maximum peak height (B) indicating a delayed response. The maximum peak on the background control had an average value of 1.26 NRLU.

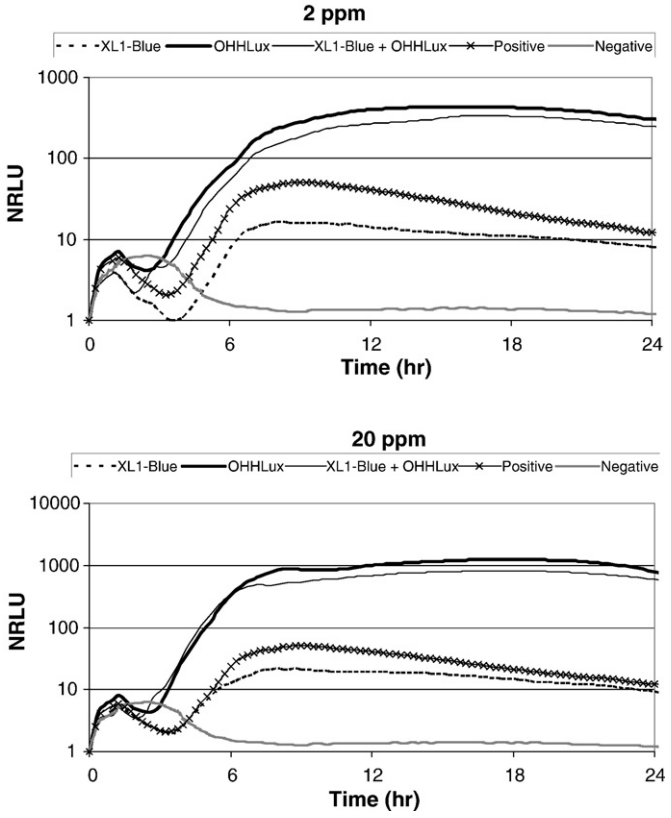

Fig. 3. Short-term disinfection effects on biosensor components. The *E. coli* biosensor 24-hour disinfection response levels in Normalized Relative Luminescence Units (NRLU) indicate a large increase in response when the bioreporter organism OHHLux was exposed to iodine. There was a slight decrease in response when XL1-Blue alone was exposed to the disinfectant. All concentrations were fixed; λ_{luxI} phage (1×10^9 pfu/mL), XL1-Blue (1×10^8 cfu/mL), and OHHLux (1×10^6 cfu/mL). The background control had an average value of 6.24 NRLU for the maximum peak.

bacteriophage were serially diluted in sterile SM buffer and plated with XL1-Blue in 3 mL Top agar in triplicate and incubated at 37 °C overnight.

Long-term starvation of the target organism, *E. coli* XL1-Blue, was conducted over 14 days. Microbial sampling and bioluminescent measurements were conducted on days 0, 1, 3, 7, and 14 during the starvation period. To create the starvation environment, 100 mL cultures of XL1-Blue were grown as previously described, rinsed and resuspended three times in equal volumes of minimal salts medium (MSM) (10 min centrifugations at 3000 ×g). MSM consisted of 7 g K_2HPO_4 (Sigma), 3 g KH_2PO_4 (Sigma), 0.1 g $MgSO_4$ (Sigma), 0.5 g $(NH_4)_2SO_4$ (Sigma), 0.01 g $CaCl_2$ (Sigma), 0.005 g $FeSO_4$ (Sigma), 0.0025 g $MnSO_4$ (Sigma), 0.0025 g Na_2MoO_4 (Sigma), adjusted to 1 L water. Cultures were maintained at room temperature with shaking at 100 rpm and transferred from the flask to a microtiter plate on days 0, 1, 3, 7, and 14 for bioluminescent assay measurements as described in the standard assay format. Within 1 day, the biosensor was registering a significant decrease in maximum peak height (ANOVA, $p=0.04$) and within 3 days it was able to detect a significant increase in the time to maximum peak height (ANOVA, $p=0.01$) in the highest *E. coli* XL1-Blue dilutions (Fig. 1). At 14 days, the biosensor was still able to respond to the target organism down to 1 cfu/mL with a 2σ or greater signal above background.

Since lactonases may be present or even common in the environment, *Arthrobacter globiformis* KACC 10580, a lactonase-producing bacterium, was used to create a mixed culture environment that could potentially inactivate the autoinducer (Park et al., 2003) and interrupt signal generation. *A. globiformis* was grown to an OD_{600} of 0.6 (~1×10^8 cfu/mL) in LBS at 30 °C and then serially diluted down to 1 cfu/mL. Each *A. globiformis* dilution (100 μL), ranging in concentration from 1×10^8 to 1 cfu/mL, was combined in microtiter plate wells with *E. coli* XL1-Blue (100 μ L), 100 μ L of λ_{luxI} phage, and 50 μL of OHHLux bioreporter cells and monitored for bioluminescence over 24 h. The system was able to autonomously respond to lambda phage infection events at target *E. coli* XL1-Blue concentrations within a co-culture of *A. globiformis* at concentrations ranging from 1×10^8 to 1 cfu/mL within the 24 h assay duration showing a reverse gradient indicating the impact of phage adhering to additional target bacteria, but not infecting it (Fig. 2).

Disinfection effects were evaluated to simulate the exposure of target cells to potable water system iodine concentrations and to quantify the impact on the phage-based biosensor assay system (Gibbons et al., 1990). *E. coli* XL1-Blue and OHHLux cultures (100 mL) were grown in LBS as described in the standard assay format, washed and resuspended in an equal volume of LBS plus iodine at 2 and 20 ppm. Cultures were maintained at room temperature with shaking at 100 rpm over a 7 day period. On days 0, 1, 3, and 7, samples were removed from the flasks and placed in microtiter plates for bioluminescent assay measurements. Disinfection at 2 ppm (ANOVA, $p=2.82\times10^{-35}$) and 20 ppm (ANOVA, $p=1.91\times10^{-39}$) of the bioreporter organism alone produced a significant increase in response within the first 24 h of exposure indicating that the bioreporter cells had become more responsive to the autoinducer. However disinfection of the target host organism alone showed a significant decrease in response for both the 2 ppm (ANOVA, $p=2.52\times10^{-15}$) and the 20 ppm (ANOVA, $p=2.18\times10^{-9}$) exposure levels (Fig. 3). The biosensor response level was increased by a minimum of an order of magnitude above the background response when both the target organism and the bioreporter were exposed to iodine. This event increased as the concentration of iodine increased and could prove useful in the future indicating that the system is more responsive during the first day of exposure to iodine. Disinfection of the target organism, XL1-Blue, at the 2 ppm (ANOVA, $p=4.41\times10^{-34}$) and 20 ppm (ANOVA, $p=2.85\times10^{-34}$) levels did significantly lower the response level of the biosensor system out to the 1 week exposure time point, however the response was 2σ or greater above the negative control indicating a positive response.

In conclusion, the *E. coli* biosensor was capable of generating a bioluminescent signal significantly above background when the target organism XL1-Blue was subjected to starvation for 2 weeks and when subjected to a lactonase containing mixed culture. The biosensor was also able to produce a detectable signal when the target organism had been exposed to iodine at 2 and 20 ppm for 1 week. However, when both the target and bioreporter organisms were exposed to iodine for up to 24 h, the bioreporter system produced a significant increase in signal response. The ability of the *E. coli* biosensor to withstand exposure to several environmental stresses over time demonstrated that the system may have a wide range of practical applications.

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