To the Graduate Council:

I am submitting herewith a thesis written by Courtney Marie Johnson entitled “Development of a Bacteriophage Based Bioluminescent Bioreporter System for the Detection of Escherichia coli K12 and O157:H7.” I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

Gary Sayler, Major Professor

We have read this thesis and recommend its acceptance:

________________________
Steve Ripp

________________________
Todd Reynolds

________________________
David Golden

Accepted for Council:

________________________
Carolyn R. Hodges, Vice Provost and
Dean of the Graduate School

(Original signatures are on file with official student records.)
Development of a Bacteriophage Based Bioluminescent Bioreporter System for the Detection of *Escherichia coli* K12 and O157:H7

A Thesis presented for the

Master of Science Degree

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Courtney Marie Johnson

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Detection of pathogenic bacteria in the environment and food products is of increasing importance especially in light of recent outbreaks of *Escherichia coli* O157:H7. I describe here a bacteriophage based bioluminescent bioreporter method for *E. coli* O157:H7 detection that combines the specificity bacteriophage have for their host, quorum sensing, and *lux* based bioluminescence from *Vibrio fischeri*. This new method for detection of *E. coli* utilizes the *luxI/luxR* quorum sensing present in *V. fischeri* which uses *N*-(*3-oxohexanoyl*)-L-homoserine lactone (OHHL) as an autoinducer (Miller & Bassler 2001). Once the concentration of OHHL is high enough it binds the LuxR protein initiating transcription of *luxCDABE* and additional *luxI*, leading to the production of light. This bioluminescent bacteriophage bioreporter method uses 2 components, first, the bioreporter cell called *E. coli* OHHLux which carries the complete *lux* cassette (*luxCDABE*) along with the transcriptional regulator *luxR* (Ripp et al. 2006). The OHHLux (lambda phage resistant) detects the diffusible OHHL produced by the second component, the *luxI* engineered phage which when infecting target *E. coli* cells produces autoinducer.

The initial feasibility of the phage based reporter assay was tested using the well characterized lambda phage and *E. coli* K12 model (Ripp et al. 2006). Once the initial feasibility of this bacteriophage based bioluminescent bioreporter system was confirmed, an *E. coli* O157:H7 specific phage, PP01, was constructed with a *luxI* insert. This PP01-*luxI* phage was used in conjunction with *E. coli* OHHLux to test for *E. coli* O157:H7 in pure culture, apple juice, ground beef, tap water, and spinach rinsates. This method has
the potential to be a sensitive, rapid, and non-invasive method of screening for *E. coli* O157:H7 contamination. The system provided rapid and sensitive detection with results in well under 24 h even at *E. coli* O157:H7 concentrations as low as 1 CFU/ml (Brigati et al. 2007, Ripp Submitted 2007). Our system is self-sufficient and could be adapted to be fully-automated and capable of high throughput screening in a production line setting.
Abstract

Detection of pathogenic bacteria in the environment and food products is of increasing importance especially in light of recent outbreaks of *Escherichia coli* O157:H7. Current detection methods for this bacterium can be sensitive and specific although they are usually costly, time consuming, and require skilled lab personnel to carry them out. I describe here a sensitive, rapid, and non-invasive method for *E. coli* O157:H7 detection that combines the specificity bacteriophage have for their host, quorum sensing, and *lux* based bioluminescence from *Vibrio fischeri*.

The *lux* genes produce multiple enzymes that carry out a complex set of reactions leading to the emission of visible light at approximately 490 nm. The *lux* genes include a cassette of five genes (*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 the transcription of the *luxCDABE* cassette (Meighen 1991). 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 bioluminescence reaction. Previously developed bacteriophage based bioluminescent bioreporters carry the *luxAB* genes, but lack the *luxCDE* genes (Ulitzur & Kuhn 1987, Stewart et al. 1989, Kodikara et al. 1991, Chen & Griffiths 1996, Loessner et al. 1996, Waddell & Poppe 2000). Thus, once the recombinant bacteriophage infects the target cell, detection requires the addition of exogenous substrate (the aldehyde component). Since the entire *luxCDABE* cassette is
too large to be accommodated by most phage genomes, a different approach is necessary to abolish the need for exogenous substrate.

This new method for detection of *E. coli* utilizes a bacterial quorum sensing system. Quorum sensing is a type of cell-to-cell communication between bacteria based on the synthesis of autoinducer molecules which trigger changes in gene expression at high concentrations. In this research, *luxI/luxR* quorum sensing present in *V. fischeri* was employed which uses *N*-(*3-oxohexanoyl*)-**L**-homoserine lactone (OHHL) as an autoinducer (Miller & Bassler 2001). *V. fischeri* have low level basal expression of *luxI* which generates small quantities of this diffusible OHHL. When enough *V. fischeri* cells are present a threshold is reached where the concentration of OHHL is high enough to bind the LuxR protein initiating transcription of *luxCDABE* and additional *luxI*, leading to the production of light. This bioluminescent bacteriophage bioreporter method uses the complete *lux* cassette (*luxCDABE*) along with the transcriptional regulator *luxR* which is carried by the bioreporter cell called *E. coli* OHHLux (Ripp et al. 2006). OHHLux (lambda phage (λ) resistant) detects the diffusible OHHL produced by the phage infected *E. coli* cells.

The initial feasibility of the phage based reporter assay was tested using the well characterized λ phage and *E. coli* K12 model (Ripp et al. 2006). A lambda phage was engineered to contain the *luxI* gene from *V. fischeri* in its genome. This reporter phage, λ*luxI*, was used as a general *E. coli* detection method. This initial assay proved to be capable of rapidly detecting *E. coli* at very low population densities. In pure culture, 1 CFU/ml was detected within 10.3 h with an additional preincubation step of 5 h. Lettuce
washings were also artificially contaminated with *E. coli* at concentrations ranging from $1 \times 10^8$ to 130 CFU/ml yielding bioluminescence within 2.6 to 22.4 h, respectively, including an additional preincubation step required at lower initial cell densities.

Once the initial feasibility of this bacteriophage based bioluminescent bioreporter system was confirmed, an *E. coli* O157:H7 specific phage, PP01, was similarly constructed with a *luxI* insert. This PP01-*luxI* phage was used in conjunction with *E. coli* OHHLux to test for *E. coli* O157:H7 in pure culture, apple juice, ground beef, tap water, and spinach rinsates. This method has the potential to be a viable option of screening for *E. coli* O157:H7 contamination. The system provided rapid and sensitive detection with results in well under 24 h even at *E. coli* O157:H7 concentrations as low as 1 CFU/ml (Brigati et al. 2007, Ripp Submitted 2007). Our system is self-sufficient and could be adapted to be fully-automated and capable of high throughput screening in a production line setting.
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Chapter I: Introduction
Bioluminescence and *Vibrio fischeri* symbiosis

Many organisms including bacteria are known to produce light in a process called bioluminescence. Perhaps some of the most well studied bioluminescent bacteria are those of the genus *Vibrio*. For example, *Vibrio fischeri*, a bioluminescent bacterium, has long been a focus of research due to its symbiotic relationship with *Euprymna scolopes* or as it is more commonly known, the Hawaiian bobtailed squid (Ruby 1999, Visick & McFall-Ngai 2000, Nyholm & McFall-Ngai 2004). *V. fischeri*, a marine bacterium, exists in a free-living planktonic state in the world’s oceans. This bacterium is known to colonize the light organ of the squid where they grow to very high densities (10^{10} to 10^{11} cells/ml) in this nutrient rich environment (Nealson & Hastings 1979, McFall-Ngai & Ruby 1991, Ruby & McFall-Ngai 1992, Ruby 1996, Graf & Ruby 1998, Nyholm & McFall-Ngai 1998).

Interestingly, the bioluminescence of *V. fischeri* is induced only when the population has reached a certain density. This process, called quorum sensing, was first discovered and described in *V. fischeri* and *V. harveyi* (Nealson et al. 1970, Eberhard 1972, Nealson & Hastings 1979). As the *Vibrio* cells grow they produce an autoinducer that accumulates as the population rises. Once the autoinducer, in the case of *V. fischeri*, the acyl homoserine lactone N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), reaches a critical threshold bioluminescence genes are induced, producing light.

The light organ of *E. scolopes* provides an ideal environment for *V. fischeri* to flourish leading to production of OHHL in a confined area (where concentrations can exceed 100 nM) leading to light production (Boettcher & Ruby 1995). Bioluminescence
from the light organ has been suggested to help the squid avoid predation. The squid is nocturnal and the luminescence produced by the bacterium provides a way to project light downward so it does not appear as a dark silhouette against the moonlight when it swims through the water, thereby camouflaging itself and making it less of a target to predators (Ruby & McFall-Ngai 1992, Ruby 1996, Graf & Ruby 1998, Ruby 1999, Visick et al. 2000, Visick & McFall-Ngai 2000, Nyholm & McFall-Ngai 2004). Each morning the squid expels a large population of *V. fischeri* from its light organ in a process known as "venting". This is hypothesized to prevent overgrowth of bacteria in the light organ and provide the source of free-living bacteria for newly hatched squids allowing the cycle to continue (Visick & McFall-Ngai 2000).

**Bioluminescence reaction**

The luminescence produced by *V. fischeri* is due to a set of genes called *lux*. The basis of the *lux* reaction consists of proteins encoded for by the *luxCDABE* operon (Meighen 1994). The *luxC*, *luxD*, and *luxE* genes produce a reductase, thioesterase, and synthetase that form a multienzyme fatty acid reductase complex (Meighen 1993). The complex diverts tetradecanoic acid from the fatty acid biosynthesis pathway generating tetradecanal, a long chain fatty aldehyde (Boylan et al. 1985, Boylan et al. 1989, Meighen 1991, Meighen 1993). The *luxA* and *luxB* genes encode for the α and β subunits of a heterodimeric luciferase enzyme (Engebrecht & Silverman 1984, Meighen 1991, Meighen 1993). The luciferase catalyzes the oxidation of reduced flavin mononucleotide (FMNH₂) along with a long chain fatty aldehyde and O₂ resulting in emission of blue

\[ \text{FMNH}_2 + \text{RCHO} + \text{O}_2 \rightarrow \text{FMN} + \text{RCOOH} + \text{H}_2\text{O} + \text{hv} \ (490 \text{ nm}) \].

After the reaction LuxCDE regenerates the aldehyde substrate and a gene called \( \text{luxG} \) is thought to encode a flavin mononucleotide reductase that produces more \( \text{FMNH}_2 \) substrate (Meighen 1993, Zenno & Saigo 1994).

**Quorum sensing**

The quorum sensing mechanism that alters gene expression leading to bioluminescence in *V. fischeri* was initially described by Nealson and Eberhard as the production, release, and detection of signal molecules called autoinducers by a population of bacteria as it increases in density (Nealson et al. 1970, Eberhard 1972, Nealson & Hastings 1979). Once the autoinducer (and by proxy the population) reaches a certain threshold concentration the bacteria respond through a change in gene expression. The functions controlled by quorum sensing are numerous and varied and include virulence, biofilm formation, antibiotic production, motility, sporulation, bioluminescence, even things like competence and conjugation are activated when an adequately large population or quorum is reached (Miller & Bassler 2001).

There are several types of quorum sensing that have been identified, each using a different type of signaling molecule as autoinducer. Both gram positive and gram
negative bacteria use furanosyl borate diester as an autoinducer for their AI-2 system. Gram positive bacteria use oligopeptides in a two component system for quorum sensing. Lastly, but most pertinent to this paper is the use of acyl homoserine lactones (AHLs) by gram negative bacteria for the LuxI/R quorum sensing mechanism.

A broad range of organisms in subclasses α, β, and γ of Proteobacteria are known to use LuxI/R quorum sensing for intraspecies communication (Camara et al. 2002, Daniels et al. 2004). The LuxI/R system is composed of two major components: (1) LuxI which synthesizes the autoinducer and (2) LuxR which binds the signaling molecule (Federle & Bassler 2003, Taga & Bassler 2003, Henke & Bassler 2004, Parsek & Greenberg 2005, Shiner et al. 2005, Reading & Sperandio 2006). As the bacteria grow they release autoinducers which accumulate as the population rises. Short-chain AHLs, like OHHL from *V. fischeri*, freely diffuse across the cell membrane while AHLs with longer acyl chains (e.g., the 12-carbon AHL of *Pseudomonas aeruginosa*) must be transported actively with efflux or influx pumps (Kaplan & Greenberg 1985, Evans et al. 1998, Pearson et al. 1999). This is due to the fact that long hydrophobic side chains decrease permeability through cell membranes. Once the autoinducer reaches a threshold level it binds to LuxR and activates the receptor protein which acts as a transcriptional activator or repressor controlling the expression of quorum sensing related genes. In the specific case of *V. fischeri*, the AHL autoinducer (OHHL) is produced which binds LuxR leading to the transcription of the *luxCDABE* cassette and producing bioluminescence.
**Organization of lux genes**

The *lux* genes in *V. fischeri* are located on two separate operons that are transcribed in opposite directions with an intergenic regulatory region called the *lux* box separating them as shown in Figure 1. The genes encoding the proteins needed for the luminescence reaction, *luxCDABEG*, and the *luxI* gene which encodes for the quorum sensing autoinducer OHHL, are located together on the same operon (Engebrecht & Silverman 1984). The *luxR* gene that encodes for the transcriptional activator that initiates luminescence after binding OHHL is separate from and in a different orientation than the rest of the *lux* genes (Engebrecht & Silverman 1987).

**Regulation of *V. fischeri* bioluminescence**

The *luxICDABEG* operon has a low basal level of transcription when *V. fischeri* cell population is low. This low transcriptional level leads to low concentrations of the OHHL autoinducer which means very little luminescence is produced (Nealson et al. 1970, Eberhard 1972). As OHHL concentration increases in correlation with cell density a critical threshold of 10 nM is reached and at this threshold OHHL attaches to the N-terminal of the LuxR binding domain causing a conformational change and dimerization.

**Acyl Homoserine Lactones (AHLs)**

LuxI-type proteins synthesize acyl homoserine lactones which are used for intraspecies communication. Homoserine lactones are chemical signals involved in microbiological quorum sensing and lead to changes in gene expression. AHLs are used as autoinducer molecules in the LuxI/R type quorum sensing system of gram negative bacteria as previously discussed. *V. fischeri* OHHL was the first AHL discovered
(Eberhard et al. 1981). Of most importance here is that the presence of high concentrations of AHLs leads to transcription of the lux genes in V. fischeri thereby generating bioluminescence. Although this is our focus, similar systems are found in other bacteria which control expression of a multitude of genes.

Structure

AHLs consist of an acyl side chain linked to a homoserine lactone ring via an amide bond. The homoserine lactone ring is common among all AHLs. However, depending on the bacterial species these molecules differ in the length of their acyl side chain (4 to 18 carbons), designated in Figure 2 by ( )n (Marketon & Gonzalez 2002). Interestingly, most AHLs discovered have an even number of carbons in their side chains. Acyl chains may also be saturated or unsaturated as seen in the examples in Table 1 (Gray et al. 1996, Schripsema et al. 1996, Puskas et al. 1997). In addition, the chain can contain an oxo, hydroxyl or no substitution on the third carbon atom, designated as R in Figure 2. Although many gram negative bacteria produce AHL there are some well known pathogenic ones that do not, for example: Escherichia coli, Haemophilus influenza, Klebsiella pneumonia, Salmonella spp., Vibrio cholerae, Campylobacter jejuni, and Helicobacter pylori (Swift 1999).

![AHL structure]

**Figure 2. AHL general structure**
<table>
<thead>
<tr>
<th>Organism</th>
<th>AHL Type</th>
<th>AHL Structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. fischeri</em></td>
<td>N-(3-oxohexanoyl)-L-homoserine lactone</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>(Eberhard et al. 1981, Engebrecht et al. 1983, Engebrecht &amp; Silverman 1984)</td>
</tr>
<tr>
<td>Organism</td>
<td>AHL Type</td>
<td>AHL Structure</td>
<td>Reference</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------------------</td>
<td>-------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><em>Ralstonia solanacearum</em></td>
<td>N-octanoyl-L-homoserine lactone</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>(Flavier et al. 1997)</td>
</tr>
</tbody>
</table>
Some bacterial species have been shown to produce more than one type of AHL although only one acts as the main quorum sensing signal (Winson et al. 1995, Schaefer et al. 1996b, Fuqua 1999, Parsek et al. 1999, Marketon et al. 2002). These secondary AHLs act as competitors against the primary AHL signal by binding the LuxR and homologue proteins in place of the primary AHL without actually activating the protein (Eberhard et al. 1986, Kuo et al. 1996, Schaefer et al. 1996a, Zhu et al. 1998). Research has suggested that AHL specificity stems from its acyl side chain, thus identification of the correct acyl-acyl carrier protein (acyl-ACP) by the LuxI protein leads to specificity in AHL production (Fuqua 1999). Structural studies indicate that the size of the LuxI-type protein cavity which interacts with the acyl side chain may be responsible for the selectivity of certain acyl-ACPs, i.e. larger cavity equals longer AHLs and vice versa (Watson et al. 2002, Gould et al. 2004). Since these molecules can be so similar they may show overlapping signal specificity. Thus a compound closely related to a bacterium’s native autoinducer can cause weak gene expression and even compete with the native AHL.

**Synthesis**

AHL molecules are synthesized by the LuxI protein and other homologues. LuxI proteins produce AHLs using metabolic substrates in the cell. When the luxI genes are transformed into a non-native host they can still produce AHL (Engebrecht et al. 1983, Pearson et al. 1994, Pearson et al. 1995, Throup et al. 1995, More et al. 1996). This is a requirement for our system since *E. coli* is being used to produce OHHL for pathogen detection and not the native host for the gene, *V. fischeri*. 
LuxI and its homologues vary greatly in gene size and protein sequence (Swift et al. 1996, Swift et al. 2001, Watson et al. 2002). When compared, protein sequences show only ten conserved amino acids mostly in the amino-terminal region of the protein, seven of which have a charge (Parsek et al. 1997, Fuqua 1999). Mutation of the amino-terminus of LuxI resulted in loss of function with five of the amino acids mutated being conserved across all known LuxI-type proteins (Arg25, Glu44, Asp46, Asp49 and Arg70) (Parsek et al. 1997). This previous research implies that the amino-terminal region and specifically these highly conserved residues may be responsible for the function of LuxI-type proteins while the variable carboxy-terminal regions may be involved in recognition of acyl-ACPs side chains (Parsek et al. 1997).

Research has determined that S-adenosylmethionine (SAM) and acyl-ACPs are the substrates involved in AHL synthesis (Schaefer et al. 1996a, Schaefer et al. 1996b, Val & Cronan 1998, Hoang et al. 1999, Hoang & Schweizer 1999, Parsek et al. 1999). SAM, a common cellular amino acid substrate, is used by LuxI to produce the homoserine lactone ring of AHL (Green 1996). The acyl side chain is acquired from acyl-ACP, a fatty acid biosynthesis metabolite. Specifically, 3-oxohexanoyl-ACP is used for synthesis of OHHL. There are several steps involved in LuxI production of AHL. The first step involves LuxI binding to SAM (Schaefer et al. 1996b, Parsek et al. 1999, Watson et al. 2002). Next, the acyl-ACP forms an amide bond with SAM yielding the acyl side chain of the AHL. Lastly, the SAM carboxylate oxygen forms a bond with the γ carbon of SAM causing lactonization resulting in the homoserine lactone ring (Schaefer et al. 1996b, Parsek et al. 1999, Watson et al. 2002).
General bacteriophage characteristics

Bacteriophage are viruses that are capable of infecting and replicating in a bacterial host. Since their discovery in the early 20th century, phage have been studied extensively especially those such as lambda. Bacteriophage are ubiquitous in the environment being found in most environments colonized by bacteria such as soil, sewage, and water. The structure of bacteriophage is comprised of a DNA or RNA genome which is contained in a protein coat. Phage are highly diverse and can have host ranges as diverse as a bacterial genera to as specific as a single bacterial species or strain. This specificity is mediated through the phage tail fibers. A bacteriophage recognizes its host via highly specific binding to receptors found on the cell surface of the bacterium. These receptor proteins are found on the outer membrane and can include things such as lipopolysaccharides, teichoic acid, flagella, pili and capsules (Heller 1992). Once the phage attaches to the bacterial cell it injects its nucleic acid into the host whose cellular machinery it will use for replicating and forming new progeny. Bacteriophage can follow one of two life cycles, lytic or lysogenic. A lytic phage will inject its genome into the host, use the bacterium’s genetic machinery to assemble numerous mature phage particles, and then proteins which lyse the cell release the bacteriophage. Alternatively, a lysogenic life cycle consists of integration of the phage genome into the host chromosome producing a prophage. The lysogenic phage will be replicated and passed with the division of the bacterium. Lysogenic phage may switch to a lytic cycle if certain environmental conditions are present such as cell damage.
**E. coli O157:H7**

Recent outbreaks of disease due to pathogenic bacteria have pushed the need for monitoring of food and water supplies to the forefront. Shiga-toxin (Stx) producing *E. coli* (STEC), specifically the enterohemorrhagic *E. coli* (EHEC), have been a major culprit in many cases. EHEC *E. coli* can cause a variety of diseases like diarrhea, hemorrhagic colitis (HC), and in some cases hemolytic uremic syndrome (HUS) and central nervous system abnormalities. Infection by EHEC can lead to very serious disease and even prove deadly particularly in children, elderly, and immunocompromised individuals. STEC expressing somatic (O) antigen 157 and flagellar (H) antigen 7 is the serotype most frequently isolated from human beings and most commonly associated with severe human disease. *E. coli* O157:H7 is the major serotype responsible for outbreaks.

al. 1998, CDC 1999, Paunio et al. 1999, Olsen et al. 2002, Samadpour et al. 2002, Bopp et al. 2003, Bruce et al. 2003), and recently spinach (CDC 2006). Given the obvious public health importance of this pathogen, a detection method that is rapid, specific, and capable of detection at low concentrations is needed.

**Traditional detection methods**

A rapid and reliable method of detection for *E. coli* O157:H7 in food and water is required to quickly detect outbreaks and hopefully limit the number of individuals affected. Currently, there are a variety of methods available for the detection of *E. coli* O157:H7 but most are slow and/or require a significant amount of sample manipulation (Deisingh and Thompson, 2004; Gehring et al., 2004; Lee et al., 2005; Moxley, 2003). The most common method used for identifying *E. coli* O157:H7 is culturing on Sorbitol MacConkey agar (SMAC) which consists of bile salts, the carbohydrate source sorbitol, and a pH indicator (Farmer & Davis 1985). SMAC can differentiate bacteria based on their ability to ferment sorbitol. Fermentation of sorbitol will produce acid, dropping the pH of the media and resulting in a color change around the colony. Most *E. coli* strains (95%) are sorbitol positive meaning they have the capacity to ferment sorbitol, whereas O157:H7 strains are not able to use this sugar. *E. coli* O157:H7 will appear as colorless colonies because large amounts of acid are not being produced while other Enterobacteriaceae appear as pink colonies due to the color change of the pH indicator. Plating methods can also involve enrichment steps before plating on SMAC plates which can increase the sensitivity of *E. coli* O157:H7 detection (Chapman et al. 1994,
Overall, plating methods can be effective in identifying *E. coli* O157:H7 but require 24-48 h of incubation, not including enrichment steps, for results to be obtained (Moxley 2003, Deisingh & Thompson 2004).

Immunological methods and Enzyme-Linked ImmunoSorbent Assays (ELISAs) have also been employed for the detection of *E. coli* O157:H7 (Moxley 2003, Deisingh & Thompson 2004, Gehring et al. 2004). These methods focus on the *E. coli* O157 and H7 antigens. Commercially available ELISAs for O157 and H7 antigens are widespread and are fairly sensitive and rapid. A Direct Immunofluorescence (DIF) assay for the detection of *E. coli* O157 was developed by Park et al. (1994). DIF consists of using human fecal samples stained with fluorescein conjugated anti-*E. coli* O157 antibodies which are examined microscopically. Immunological methods do have several drawbacks: (1) numerous staining and washing steps (Moxley 2003, Deisingh & Thompson 2004, Gehring et al. 2004) (2) false negatives for H7 because of culturing methods and (3) O157 false positives seen with *Citrobacter freundii*, *E. hermannii*, and *Salmonella urbana* (Park et al. 1994, Dylla et al. 1995). Immunogenic separation of target cells can however prove useful for lowering detection limits/times and is now being integrated into assays (Goodridge et al. 1999, Favrin et al. 2001, Su & Li 2004, Fu et al. 2005, Wang et al. 2007, Ripp Submitted 2007).

Detection by Polymerase Chain Reaction (PCR) has also been used for *E. coli* O157:H7 although there are several hurdles. Due to the fact that there are many inhibitors found in feces and food samples, PCR is most commonly used to confirm isolates as *E. coli* O157:H7 but it can also be used to directly detect the organism. To
ensure there are no false positives and that *E. coli* O157:H7 is the only organism that will produce the desired amplification, multiplex PCR is often employed. Multiplex PCR is a variation of normal PCR, in which several targets of interest are simultaneously amplified in one reaction, using more than one pair of primers. Hu et al. (1999) used the first successful multiplex PCR to specifically detect *E. coli* O157:H7 serotype. This multiplex PCR amplified several targets: (1) *eaeA*<sub>O157</sub>, the gene encoding for intimin which is involved in the attaching and effacing adherence phenotype (2) *stx1* and *stx2*, the genes responsible for Shiga toxin production, (3) *filC*, the H7 flagellar antigen gene and (4) *rfbE*, which encodes the *E. coli* O157 serotype. Hu et al. (1999) successfully detected target organisms in isolated colonies, however, attempts directly from feces failed without enrichment. PCR is rapid and sensitive considering that theoretically DNA from a single bacterial cell can be amplified in about 1 h for a positive result if no enrichment is necessary. Problems with the PCR method include its complexity, possible contamination, inability to distinguish between live and dead bacterial cells, and the fact that food and environmental samples often contain PCR inhibitors (Deisingh & Thompson 2004).

Quantitative PCR (Q-PCR) which allows simultaneous amplification, via PCR, and quantification of targeted DNA or RNA molecules, via fluorescent probes, has also been applied as an *E. coli* O157:H7 assay. Target sequences ranged from *eaeA*, *stx*, to *rfbE* either alone or in a multiplex format and concentrations down to 1 CFU/ml or g were detected in relatively short periods of time (< 8 h including enrichment) (Meng et al. 1996, Fortin et al. 2001, Jothikumar & Griffiths 2002, Sharma & Dean-Nystrom 2003, Fu
et al. 2005, Hsu et al. 2005, Holicka et al. 2006, Yoshitomi et al. 2006). This is a viable method for sensitive, rapid and quantitative way to screen for \textit{E. coli} O157:H7. Although, just like all other methods Q-PCR has its limitations, the necessary equipment and reagents are costly. As with basic PCR, Q-PCR is complex, subject to contamination, detects viable but non-culturable cells (VBNC) and free DNA, and inhibitors present in the complex samples may affect assay reliability.

**Bioluminescent bioreporters**

Bioluminescent bioreporters are organisms genetically engineered to react to the presence of a specific analyte by generation of light. Bioreporters are constructed by placing a promoter which is specifically induced by a particular analyte upstream of the reporter genes, in our case the \textit{lux} bioluminescence genes. This construct can be contained in the host bacterium as a plasmid or can be integrated into the chromosome. They are often a rapid, non-invasive, sensitive, and specific alternative to other detection methods. Bioluminescent bioreporters can be used to determine bioavailability along with possible toxic effects produced by chemicals on organisms.

There is a long history of bioluminescent bioreporters with detected analytes including environmental pollutants, toxic chemicals in wastewaters, bacterial nutrients, and pathogens. Examples of these bioreporters and their detection limits are shown in Table 2. These bioreporter systems all use the \textit{V. fischeri} luxCDABE genes although other common bioluminescence reporter genes from \textit{Photorhabdus luminescens} and \textit{V. harveyi}
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3 Dichlorophenol</td>
<td>50 mg/L</td>
<td>(Hay et al. 2000)</td>
</tr>
<tr>
<td>3-Xylene</td>
<td>3 μM</td>
<td>(Burlage 1998)</td>
</tr>
<tr>
<td>4-Chlorobenzoate</td>
<td>380 μM – 6.5 mM</td>
<td>(Rozen et al. 1999)</td>
</tr>
<tr>
<td>Alginate production</td>
<td>50 – 150 mM NaCl</td>
<td>(Wallace et al. 1994)</td>
</tr>
<tr>
<td>Ammonia</td>
<td>20 μM</td>
<td>(Simpson 2000)</td>
</tr>
<tr>
<td>Antibiotic effectiveness against</td>
<td>100 CFU</td>
<td>(Francis et al. 2000)</td>
</tr>
<tr>
<td>Staphylococcus aureus in mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BTEX (benzene, toluene, ethylbenzene, xylene)</td>
<td>0.03 – 50 mg/L</td>
<td>(Applegate et al. 1998)</td>
</tr>
<tr>
<td>Cadmium</td>
<td>19 mg/kg</td>
<td>(Corbisier et al. 1996)</td>
</tr>
<tr>
<td>Chromate</td>
<td>10 μM</td>
<td>(Peitzsch et al. 1998)</td>
</tr>
<tr>
<td>Cobalt</td>
<td>2.0 mM</td>
<td>(Tibazarwa et al. 2000)</td>
</tr>
<tr>
<td>Copper</td>
<td>1 μM – 1 mM</td>
<td>(Holmes et al. 1994, Daunert et al. 2000)</td>
</tr>
<tr>
<td>DNA damage (mitomycin)</td>
<td>0.032 μg/ml</td>
<td>(Vollmer et al. 1997)</td>
</tr>
<tr>
<td>Gamma-irradiation</td>
<td>1.5 – 200 Gy</td>
<td>(Min et al. 2000)</td>
</tr>
<tr>
<td>Hemolysin production</td>
<td>5 mM cAMP</td>
<td>(Bang et al. 1999)</td>
</tr>
<tr>
<td>in vivo monitoring of Salmonella typhimurium infections in living mice</td>
<td>100 CFU</td>
<td>(Contag et al. 1995)</td>
</tr>
<tr>
<td>Iron</td>
<td>10 nM – 1 μM</td>
<td>(Khang et al. 1997)</td>
</tr>
<tr>
<td>Isopropyl benzene</td>
<td>1 – 100 μM</td>
<td>(Selifonova &amp; Eaton 1996)</td>
</tr>
<tr>
<td>Lead</td>
<td>4036 mg/kg</td>
<td>(Corbisier et al. 1996)</td>
</tr>
<tr>
<td>Mercury</td>
<td>0.025 nM</td>
<td>(Selifonova et al. 1993)</td>
</tr>
<tr>
<td>N-acyl homoserine lactones</td>
<td>Not specified</td>
<td>(Winson et al. 1998)</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>12 – 120 μM</td>
<td>(King et al. 1990)</td>
</tr>
<tr>
<td>Nickel</td>
<td>0.3 mM</td>
<td>(Tibazarwa et al. 2000)</td>
</tr>
<tr>
<td>Nitrate</td>
<td>0.05 – 50 μM</td>
<td>(Prest et al. 1997)</td>
</tr>
<tr>
<td>Organic peroxides</td>
<td>Not specified</td>
<td>(Belkin et al. 1996)</td>
</tr>
<tr>
<td>PCBs</td>
<td>0.8 μM</td>
<td>(Layton et al. 1998)</td>
</tr>
<tr>
<td>p-chlorobenzoic acid</td>
<td>0.06 g/l</td>
<td>(Rozen et al. 1999)</td>
</tr>
<tr>
<td>Salicylate</td>
<td>36 μM</td>
<td>(King et al. 1990)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>5 ng/ml</td>
<td>(Hansen &amp; Sorensen 2000)</td>
</tr>
<tr>
<td>Trichloroethylene</td>
<td>5 – 80 μM</td>
<td>(Shingleton et al. 1998)</td>
</tr>
<tr>
<td>Ultrasound</td>
<td>500 W/cm²</td>
<td>(Vollmer et al. 1998)</td>
</tr>
<tr>
<td>Ultraviolet light</td>
<td>2.5 – 20 J/m²</td>
<td>(Elasri &amp; Miller 1998)</td>
</tr>
<tr>
<td>Zinc</td>
<td>0.5 – 4 μM</td>
<td>(Erbe et al. 1996)</td>
</tr>
</tbody>
</table>
can be used as well. In addition, many other bioreporter systems have been constructed using only the *luxAB* genes. The disadvantage of just using *luxAB* is that only the luciferase is self generated and exogenous aldehyde substrate must be added to the assay to generate bioluminescence. The bioreporter gene product, luminescence, is easily detected and measured by light-measuring devices, such as photomultiplier tubes, photodiodes, microchannel plates or charge-coupled devices. In fact, bioreporters can be integrated into biosensors, forming devices that are a blend of biological and electrical components so that the light produced can be measured to detect and quantify the target. The properties of the bioluminescence signal produced make it ideal for construction of high throughput, continuous and real-time monitoring systems.

**Bacteriophage based bioluminescent bioreporters**

Bioluminescent bioreporters can also be adapted to be used with bacteriophage to detect bacterial pathogens. The combination of the phage based detection with bioluminescence genes provides a viable detection system (Billard & DuBow 1998, Galindo et al. 2002, Hazbon 2004, Petty et al. 2007). Phage can exhibit a high specificity for their hosts making them ideal to use for specific bacterial pathogen detection. These bacteriophage based systems can be specific, sensitive, rapid, and cost effective. A phage based system can detect living cells and those in a viable but non-culturable (VBNC) state unlike PCR and antibiotic based methods. Phage are relatively inexpensive and easy to produce in large quantities and detection of target at concentrations as low as 1 CFU/ml is possible with limited preincubation. There have been phage based
bioluminescent bioreporters created to detect bacteria such as *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *M. smegmatis*, *Salmonella* spp., *E. coli* and other enteric bacteria (Table 2). In classical phage based detection systems, the recombinant phage usually carries the bacterial *luxAB* or eukaryotic *lucAB* genes. When they infect the host organism the *lux* genes are transferred into the host cell and expressed producing the luciferase enzyme. In the presence of exogenous aldehyde substrate, this results in generation of bioluminescence which can be readily measured, determining if the target organism is present.

**E. coli O157:H7 bacteriophage based bioluminescent bioreporter system**

There are various methods available for the detection of *E. coli* O157:H7 all have similar limitations including slow results, false positives, complex procedures, and significant sample manipulations. The phage reporter method described here differs from the aforementioned methods in that it exploits the specificity that bacteriophage exhibit for their host to produce a new technique for identifying this important pathogen. The combination of *lux* bioluminescence and bacterial quorum sensing genes with a specific
<table>
<thead>
<tr>
<th>Target Pathogen</th>
<th>Phage Used</th>
<th>Genes Used</th>
<th>Sample Tested</th>
<th>Detection Time</th>
<th>Detection Limit</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em></td>
<td>A511</td>
<td><em>V. harveyi luxAB</em></td>
<td>Culture Spiked salad</td>
<td>2 h 22-24 h</td>
<td>100 - 10^3 cells/ml 1 cell/g</td>
<td>(Loessner et al. 1996)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>A511</td>
<td><em>V. harveyi luxAB</em></td>
<td>Spiked pudding, cabbage Spiked meat, cheese</td>
<td>20 h</td>
<td>1 cell/g 10-100 cells/g</td>
<td>(Loessner et al. 1997)</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>TM4</td>
<td>Firefly Luciferase (Flux)</td>
<td>Clinical isolates and sputum</td>
<td>2-3 days</td>
<td>ND</td>
<td>(Banaiee et al. 2001, Bardarov et al. 2003)</td>
</tr>
<tr>
<td><em>M. smegmatis and tuberculosis</em></td>
<td>TM4</td>
<td>Flux</td>
<td>Culture</td>
<td>24 h</td>
<td>10^4-10^5 cells</td>
<td>(Jacobs et al. 1993)</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>L5</td>
<td>Flux</td>
<td>Culture</td>
<td>20 h 40 h</td>
<td>10^3 cells/g 100 cells/g</td>
<td>(Sarkis et al. 1995)</td>
</tr>
<tr>
<td><em>S. typhimurium</em></td>
<td>P22</td>
<td>luxAB</td>
<td>Soil, water, and sewage sludge</td>
<td>24 h</td>
<td>1 cell/ml</td>
<td>(Turpin et al. 1993)</td>
</tr>
<tr>
<td><em>Salmonella sp.</em></td>
<td>P22</td>
<td><em>V. fischeri luxAB</em></td>
<td>Culture Eggs</td>
<td>6 h 24 h</td>
<td>10 cells/ml 10 cells/egg</td>
<td>(Chen &amp; Griffiths 1996)</td>
</tr>
<tr>
<td>Enteric bacteria</td>
<td>ФV10</td>
<td><em>V. fischeri luxAB</em></td>
<td>Meat</td>
<td>50 min 4 h</td>
<td>10^4 cells/g 10 cells/g</td>
<td>(Kodikara et al. 1991)</td>
</tr>
<tr>
<td><em>E. coli O157:H7</em></td>
<td>ФV10</td>
<td><em>V. harveyi luxAB</em></td>
<td>Culture</td>
<td>1 h</td>
<td>ND</td>
<td>(Waddell &amp; Poppe 2000)</td>
</tr>
<tr>
<td><em>Salmonella sp.</em></td>
<td>Felix 01</td>
<td><em>Vibrio luxAB</em></td>
<td>Culture</td>
<td>ND</td>
<td>ND</td>
<td>(Kuhn et al. 2002)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>λCharo n 30</td>
<td><em>V. fischeri luxAB</em></td>
<td>Milk and urine</td>
<td>1 h</td>
<td>10 - 100 cells/ml</td>
<td>(Ulitzur &amp; Kuhn 1987)</td>
</tr>
</tbody>
</table>
*E. coli* O157:H7 phage creates a bioreporter system for sensing O157:H7 in samples. This provides a novel detection technique that is rapid and amenable to real-time monitoring.

Our method utilizes a bacteriophage that specifically infects *E. coli* O157:H7 in combination with quorum sensing and *lux* based bioluminescence to create a phage based bioreporter system for this pathogen. Genetic manipulation of bacteriophage using reporter genes such as bacterial luciferase (*luxAB*), β-galactosidase (*lacZ*) and green fluorescent protein (*gfp*) to create “reporter phage” has been well documented (Ulitzur & Kuhn 1987, Stewart et al. 1989, Kodikara et al. 1991, Turpin et al. 1993, Chen & Griffiths 1996, Loessner et al. 1996, Waddell & Poppe 2000, Goodridge & Griffiths 2002). Since phage are metabolically inactive outside of their host, the reporter proteins encoded in these phage are not produced until the specific host bacterium is infected. In previously described *lux* based phage reporter systems, bacteria are detected after phage infection, an incubation/growth step, and the addition of an exogenous substrate (Kodikara et al. 1991, Chen & Griffiths 1996, Loessner et al. 1996). Although these current methods are able to specifically and sensitively detect bacteria, they are not amenable to continuous monitoring because of the requirement for downstream sample manipulation.

**PP01 bacteriophage background**

The phage used to develop our reporter system was a virulent bacteriophage referred to as PP01 that is specific for *E. coli* O157:H7. It was isolated and characterized
in 2002 from swine stool sample (Morita et al. 2002b). The PP01 phage infects *E. coli* O157:H7 but fails to infect other O-serogroups and the strain K-12 (Table 4) (Morita et al. 2002b). Analysis of the amino acid sequence from the tail fiber proteins Gp37 and Gp38, the end proteins in each tail fiber which are involved in attachment and determining host range, determined PP01 was related to T2 phage (Morita et al. 2002b). Numerous phage were isolated from the stool samples tested but Morita et al. (2002a) found that PP01 phage was the only isolate to decrease the OD$_{600}$ of an *E. coli* O157:H7 culture, evidence that PP01 is a lytic phage. One PP01 phage resistant *E. coli* mutant was found in the process. Analysis of the mutant showed it had lost the outer membrane protein OmpC, a protein which forms a large diameter transmembrane channel or pore in the *E. coli* cell wall but had instead increased production of an alternative porin OmpF (Schindler & Rosenbusch 1978, Nikaido & Rosenberg 1983). A 14 kb deletion upstream of and including a portion of OmpC caused the loss of the protein (Morita et al. 2002a). Complementation of OmpC from an *E. coli* O157:H7 strain (but not a K-12 strain) restored the mutant’s susceptibility to PP01 causing Morita et al. (2002a) to suggest that the OmpC protein serves as the receptor for this bacteriophage.

Recently, a group has modified this PP01 bacteriophage by labeling it with green fluorescent protein (GFP) to detect *E. coli* O157:H7 (Oda et al. 2004). The bacteriophage’s small outer capsid (SOC) protein, which plays an important role in phage capsid stability (Ishii & Yanagida 1977), was used as an insertion point for a GFP marker. The process involved creating plasmids through PCR and restriction enzyme digest that have the GFP marker inserted at either the C or N terminal region of the *soc*
Table 4. Bacterial strains used to test PP01 phage’s specificity and host range

<table>
<thead>
<tr>
<th>Host serotype or species</th>
<th>Strain</th>
<th>Susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> O6:H16</td>
<td>941-88</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> O7</td>
<td>ATCC23503</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> O55</td>
<td>992-97</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> O116:H10</td>
<td>ATCC23541</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> O119:H6</td>
<td>997-97</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> O126:H12</td>
<td>947-88</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>980013</td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>9800012</td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>EDL933</td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>ATCC43888</td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli</em> O157:H7</td>
<td>CR-3</td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli</em> BE</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> K-12</td>
<td>12017</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> K-12</td>
<td>RK4784</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em> K-12</td>
<td>W3110</td>
<td>-</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>KTY-1</td>
<td>-</td>
</tr>
<tr>
<td><em>Erwinia cartovora</em></td>
<td>MAFF301614</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>PAO1</td>
<td>-</td>
</tr>
<tr>
<td><em>Yersinia enterocolitica</em></td>
<td>ZT23</td>
<td>-</td>
</tr>
</tbody>
</table>

(taken from Morita et al. 2002b)
gene (pUC-GFP/SOC and pUC-SOC/GFP). A bacterium carrying this plasmid was then infected with wild type PP01. Homologous recombination between the plasmid and the host genome produced recombinant GFP phage which were detected through plaque hybridization assays. The fusion did not change PP01’s natural host range nor did it decrease the bacteriophage’s binding affinity for its host bacterium. The fusion did however decrease the stability of the phage in alkaline solution (Oda et al. 2004).

Oda et al. (2004) reported that this recombinant phage allows for specific detection of culturable and VBNC *E. coli* O157:H7 (2004). Detection is achieved through mixing the recombinant phage with a culture or sample. Attachment of the GFP-phage to the *E. coli* cell surface allows visualization of the bacterial pathogen even in a VBNC state under a fluorescent microscope. In addition, an increase in fluorescence intensity of the culture over time, indicating replication and increase of recombinant phage numbers, signifies a culturable form of the pathogenic bacterium is present. The PP01-GFP reporter phage does appear, at least in preliminary studies, to be rapid and specific; however like every GFP based assay it needs a high energy light source for excitation of the GFP protein which could limit its potential use in real-time online industrial and environmental monitoring. The PP01 reporter phage presented in this paper uses the bacterial *lux* reporter genes to create a construct that allows for detection without the addition of substrate and has potential for use in real-time online monitoring.
Recombinant luxI phage and E. coli OHHLux

**λ**<sub>luxI</sub> phage

The recombinant λ<sub>luxI</sub> reporter phage was developed as a proof of concept prior to the final *E. coli* O157:H7 specific PP01-luxI phage creation. The construction of λ<sub>luxI</sub> is fully described in Chapter II and Figure 5 in detail including primer sequences, vectors, and restriction enzymes used. Briefly, a plasmid carrying luxI was fused with the promoter (P<sub>L</sub>) of phage lambda upstream and the transcriptional terminator, *rrnB* T<sub>1</sub>T<sub>2</sub>, downstream to create a P<sub>L</sub>-luxI-*rrnB* construct. Next, this P<sub>L</sub>-luxI-*rrnB* was placed into the Lambda ZAP II cloning vector. The recombinant lambda phage DNA produced was then packaged into phage heads. Resulting plaques were screened using a plaque hybridization assay. Positive plaques containing the luxI incorporated phage were then isolated and propagated on top agar plates and used in subsequent assays with *E. coli* OHHLux bioreporter cells to determine the viability of their use as a screening method for *E. coli* K12.

**E. coli** OHHLux bioreporter cells

The basics of construction for *E. coli* OHHLux are as follows but see Chapter II and Figure 6 for a more detailed description including details about primer sequences, vectors, and restriction enzymes used. The OHHL-specific bioluminescent bioreporter *E. coli* OHHLux was constructed by fusing the luxCDABE genes which are responsible for the bioluminescence reaction with the luxR gene, the transcriptional activator. Along with these genes *rrnB* T<sub>1</sub>T<sub>2</sub>, the transcriptional terminator, was added to prevent readthrough resulting in aberrant light production and a kanamycin resistance gene
allowing for stability and selection of our construct in our bioreporter cell. The construction resulted in a plasmid consisting of \textit{rrnB-luxCDABE-luxR-Kn} which was used along with the PP01-\textit{luxI} phage in our \textit{E. coli} O157:H7 screening assays.

**PP01-\textit{luxI} phage**

The methods used for the construction of our recombinant PP01-\textit{luxI} reporter phage were based on those used by Oda et al. (2004). The construction of PP01-\textit{luxI} is fully described in Chapter III and Figures 11 and 12 in detail including primer sequences, vectors, and restriction enzymes used. Briefly, a plasmid carrying \textit{luxI} fused with the promoter (\textit{P}L) of phage lambda upstream and the transcriptional terminator, \textit{rrnB} \textit{T1T2}, downstream creating a \textit{P}L-\textit{luxI}-\textit{rrnB} construct. Next, \textit{soc2}, \textit{soc1} and \textit{soc} genes from the PP01 phage were cloned into the \textit{P}L-\textit{luxI}-\textit{rrnB} construct. The \textit{soc2} and \textit{soc1} genes were inserted upstream of \textit{P}L while \textit{soc} was placed downstream of \textit{rrnB} producing a \textit{soc2soc1}-\textit{P}L-\textit{luxI}-\textit{rrnB}-\textit{soc} plasmid. This plasmid was then used for homologous recombination with PP01.

Homologous recombination was accomplished by producing \textit{E. coli} O157:H7 cells containing the \textit{soc2soc1}-\textit{P}L-\textit{luxI}-\textit{rrnB}-\textit{soc} plasmid which were then infected with PP01 phage and incubated. After incubation, phage were extracted and PP01-\textit{luxI} phage were identified with a plaque hybridization assay. Positively identified PP01-\textit{luxI} phage were propagated and used in subsequent assays with \textit{E. coli} OHHLux bioreporter cells to determine the viability of their use as a screening method for \textit{E. coli} O157:H7.
**System mechanics**

As previously stated, the detection method for *E. coli* O157:H7 combines the specificity bacteriophage have for their host, quorum sensing and *lux* based bioluminescence from *V. fischeri*. How all these components function together and the steps involved in the assay follows and are depicted in Figure 3.

1.) If the target pathogen is present, the *luxI* reporter phage will specifically infect the *E. coli* and insert its genome.

2.) After infection, *luxI* will be transcribed by the host from the phage genome resulting in production of OHHL from Acyl-ACP and SAM.

3.) The OHHL diffuses into nearby bioreporter cells, *E. coli* OHHLux, and binds the transcriptional activator LuxR.

4.) The LuxR-OHHL complex interacts with P_L leading to the transcription of *luxCDABE*.

5.) LuxCDE form a multienzyme complex that generates aldehydes (by diverting myristol-ACP away from fatty acid biosynthesis pathway), while the LuxAB luciferase oxidizes FMNH_2 and a long chain fatty aldehyde resulting in a measurable light signal at 490 nm and a positive assay result.
Figure 3. Bacteriophage based bioluminescent bioreporter for pathogen detection
Chapter II: Linking bacteriophage infection to quorum sensing signaling and bioluminescent bioreporter monitoring for direct detection of bacterial agents
This is a version of an article found in the Journal of Applied Microbiology entitled “Linking bacteriophage infection to quorum sensing signaling and bioluminescent bioreporter monitoring for direct detection of bacterial agents”.

Abstract

The aim of this research was to incorporate into the lambda phage genome a luxI based acyl-homoserine lactone (AHL) synthase genetic construct and exploit the autoamplified power of quorum sensing to translate a phage infection event into a chemical signature detectable by a lux based bioluminescent bioreporter, with focus towards facile detection of microbial pathogens. The luxI gene from Vibrio fischeri was inserted into the lambda phage genome to construct a model phage based biosensor system for the general detection of Escherichia coli. The AHL signalling molecules synthesized upon phage infection are detected by an AHL specific bioluminescent bioreporter based on the luxCDABE gene cassette of V. fischeri. The assay generates target specific visible light signals with no requisite addition of extraneous substrate. This binary reporter system was able to autonomously respond to lambda phage infection events at target E. coli concentrations ranging from $1 \times 10^8$ to 1 CFU/ml within 1.5 to 10.3 h, respectively, in pure culture. When assayed against artificially contaminated lettuce leaf washings, detection within an E. coli inoculum range from $1 \times 10^8$ to 130 CFU/ml was achieved within 2.6 to 22.4 h, respectively. The initial feasibility of binary phage based reporter assays indicates that quorum sensing can be used to translate a phage infection event into an autoamplified chemical signature. With further
modification, binary phage based reporter assays may be capable of rapidly and cost-effectively detecting pathogenic agents at very low population densities.

**Introduction**

In 1987, Ulitzur and Kuhn (Ulitzur & Kuhn 1987) reported a novel pathogen detection method that coupled the specificity of bacteriophage for their unique bacterial hosts with bioluminescent signalling. They cloned the $luxAB$ encoded luciferase genes from *Vibrio fischeri* into the phage lambda genome. Upon infection, the $luxAB$ genes were transduced to *Escherichia coli*, thus endowing these host cells with a bioluminescent phenotype visible upon addition of a requisite aldehyde substrate. This technique has since been applied to other phage for specific, low-level (10 – 1000 cells) detection of *Listeria monocytogenes* (Loessner et al. 1996), *Salmonella typhimurium* (Chen & Griffiths 1996), *E. coli* O157:H7 (Waddell & Poppe 2000), enteric bacteria (Kodikara et al. 1991), and *Staphylococcus aureus* (Pagotto *et al.* 1996) within a variety of food matrices. The firefly luciferase (*luc*) (Sarkis et al. 1995), ice nucleation (*inaW*) (Wolber & Green 1990), beta-galactosidase (*lacZ*) (Goodridge & Griffiths 2002), and green fluorescent protein (*gfp*) (Funatsu et al. 2002, Oda et al. 2004) genes have similarly been incorporated into bacteriophage for the detection of foodborne pathogens such as *Mycobacterium*, *Salmonella*, and *E. coli*. Reporter phage have also been labeled with a variety of fluorescent dyes for bacterial specific tagging (Mosier-Boss *et al.* 2003) and combined with immunomagnetic separation for rapid capture, concentration, and identification of bacterial targets (Goodridge et al. 1999, Favrin et al. 2001). In addition,
bacteriophage in their unadorned native form have been used for decades in phage typing schemes to identify foodborne as well as clinical bacterial isolates (Stone 2002).

Clearly, the exploitation of phage specificity for bacterial monitoring has potential for foodborne pathogen monitoring. However, current phage assay systems require addition of substrate or specialized monitoring equipment that is not adaptable to the real-time, on-line monitoring format desired by the food industry. To move towards a fully reagentless phage based assay, we have developed a model binary recombinant phage system for the detection of bacterial pathogens that links phage infection events to quorum sensing signal molecule biosynthesis and bioluminescent bioreporter induction (Fig. 4). The system’s foundation centers on the luxCDABE operon of V. fischeri and its regulatory genes luxI and luxR (Meighen 1994). The luxAB component of this operon encodes for a bacterial luciferase that generates bioluminescence when provided with oxygen, FMNH₂, and an aldehyde substrate synthesized by the luxCDE gene complex. The luxI encoded LuxI protein is responsible for generation of a specific acyl-homoserine lactone (AHL) signalling molecule referred to as an autoinducer. Freely diffusible autoinducers interact with LuxR proteins to stimulate transcription of luxCDABE and luxI. As the concentration of AHL autoinducer increases, so does the number of LuxR binding episodes, and an autoamplified quorum sensing loop is established that results in the generation of bioluminescence in a cell density dependent manner (Whitehead et al. 2001).

In the present detection system a phage, chosen based on its specificity for the desired target bacterium, is engineered to contain the luxI gene within its chromosome. Upon infection, luxI is inserted into the host and expressed, thereby creating a cell that
The recombinant phage contains a \textit{luxI} gene that, upon infection of its specific host, is inserted into the host chromosome where it is transcribed, thereby synthesizing acyl-homoserine lactone (AHL) autoinducer. The diffusible AHL molecules interact with the LuxR regulatory protein to trigger \textit{luxCDABE} transcription in the neighboring bioluminescent bioreporter cell to generate bioluminescence. In these experiments, \textit{E. coli} XL1-Blue was used as a model bacterial pathogen.

\textbf{Figure 4. Reporter phage assay for targeted detection of bacterial pathogens}
actively synthesizes autoinducer. As the autoinducer molecules diffuse into the extracellular environment, they are detected by an AHL specific bioluminescent bioreporter that contains the luxR and luxCDABE genes (see Daunert et al. (Daunert et al. 2000) or Keane et al. (Keane et al. 2002) for a complete review of bioreporter detection systems). The interaction of autoinducer with LuxR stimulates luxCDABE expression and the bioreporter generates a light signal at 490 nm. Thus, the initial phage infection event yields an autoamplified chemical signature that is sensed and communicated through bioluminescent bioreporter signal induction. The assays are fully reagentless and amenable to high throughput, near real-time screening of bacterial pathogens. As a model system, we have incorporated luxI into phage lambda for the general detection of E. coli and constructed a luxRCDABE bioluminescent bioreporter for specific sensing of the autoinducer N-3-(oxohexanoyl)-L homoserine lactone (OHHL). The assay directly detects E. coli in pure culture at $1 \times 10^8$ CFU/ml within 1.5 h and at 1 CFU/ml, with an additional preincubation step, within 10.3 h. Washings from lettuce leaves inoculated with E. coli at concentrations ranging from $1 \times 10^8$ to 130 CFU/ml produced bioluminescence within 2.6 to 22.4 h, respectively, with requisite independent preincubation at lower initial cell densities.

Materials and methods

Bacterial strains and bacteriophage

The phage bioluminescent system consists of three components; the luxI incorporated reporter phage ($\lambda_{\text{luxI}}$), the AHL specific bioluminescent bioreporter (E. coli OHHLux), and the target bacterium. The $\lambda_{\text{luxI}}$ reporter phage was constructed within
temperate phage lambda, lambda resistant *E. coli* XLOLR (Stratagene, La Jolla, CA) was used for construction of the OHHL specific bioluminescent bioreporter *E. coli* OHHLux, and the *E. coli* K12 variant XL1-Blue (Stratagene) was used as the model host strain for phage infection. *lux* genes were derived from *V. fischeri* or *Photorhabdus luminescens* (Gupta et al. 2003). *E. coli* strains were typically grown in Luria-Bertani media (LB; 10 g tryptone, 5 g yeast extract, 10 g NaCL per l H$_2$O, pH 7.0). NZY top agar (5 g NaCL, 2 g MgSO$_4$ · 7H$_2$O, 5 g yeast extract, 10 g NZ amine, 7 g agarose per l H$_2$O, pH 7.0) was used to propagate and titer bacteriophage.

**Genetic construction of theluxI reporter bacteriophage**

The fundamental construction of the $\lambda_{luxI}$ reporter phage consisted of a fusion of the *V. fischeri luxI* gene (Accession #Y00509) upstream of the left arm promoter ($P_L$) of phage lambda in a pLEX vector (Invitrogen, Carlsbad, CA) (Fig. 5). Upstream to this fusion was ligated an *rrnBT_1T_2* transcriptional terminator from the pKK223-3 cloning vector (Accession #M77749). Each individual gene and step-wise fusions were initially constructed in pCR2.1- or pCR4-TOPO TA cloning vectors (Invitrogen) and then the entire fusion ligated into the Lambda ZAP II cloning vector (Stratagene) and packaged into phage lambda using Gigapack III Gold packaging extract (Stratagene). DNA isolations were performed with Wizard Minipreps, Midipreps, or Lambda Preps (Promega, Madison, WI) and purified when necessary with the Geneclean Spin Kit (Q-Biogene, Carlsbad, CA). PCR reactions were carried out in an MJ Research DNA Engine tetrad (Waltham, MA) using Ready-To-Go PCR beads (Amersham Piscataway, NJ). DNA was sequenced at all steps with the ABI Big Dye Terminator Cycle
Figure 5. Genetic construction scheme for the \( \lambda_{luxI} \) reporter bacteriophage
Sequencing reaction kit on an ABI 3100 DNA Sequencer (Perkin-Elmer, Foster City, CA).

The *luxI* gene was PCR-amplified from *V. fischeri* using the primer pairs 5'-CATATGACTATAATGATAAAAAAATCGG-3' and 5'-CATATGTAAATTTAAGACTGC-3' to introduce the restriction site *NdeI* at both termini (underlined) and cloned into a pCR2.1-TOPO vector (Fig. 5A). The *luxI* gene was then removed from the TOPO vector by *NdeI* restriction digestion and ligated into the *NdeI* multicloning site (MCS) of the pLEX vector, thereby placing *luxI* in frame with the *P_L* promoter (Fig. 5B). Directionality was confirmed by restriction digestion and sequencing.

The *rrnB* transcriptional terminator was PCR-amplified from pKK223-3 using the primer pairs 5'-ATCGATAAGAGTTTGTAGAAACGC-3' and 5'-CTGTTTTGGCGGATG-3' to introduce the restriction site *ClaI* at the 5' end (underlined) and cloned into a pCR4-TOPO vector (Fig. 5C).

The *P_L-luxI* fusion was PCR-amplified out of pLEX with the primer pairs 5'-ATCGATGTCGACTCTAGAGGATCC-3' and 5'-ATCGATATTGACGCTCGGTACCATA-3' containing the restriction sites *ClaI* (underlined) and cloned into a pCR2.1-TOPO vector (Fig. 5D). This vector was then digested with *ClaI* and ligated into the *ClaI* site of the *rrnB* TOPO vector described above to create a *P_L-luxI-rrnB* fusion within a pCR4-TOPO vector (Fig. 5E). Directionality was again confirmed by restriction digestion and sequencing. Unique *EcoRI* sites within the multicloning site of the pCR4-TOPO vector now flanked the *P_L-*
**luxI-rrnB** fusion, allowing for its removal via *Eco*RI digestion with subsequent ligation into the unique *Eco*RI site of the Lambda ZAP II vector (Fig. 5F). Resulting recombinant lambda phage DNA was then packaged into phage heads using Gigapack III packaging extract per the manufacturer’s instructions (Stratagene). Resulting plaques were hybridized with an alkaline phosphatase labeled *luxI* probe to chemifluorescently identify *luxI* incorporated phage using an Alkphos Direct Labeling and Detection kit (Amersham). Positive plaques were then isolated and propagated on top agar plates as described in the Lambda ZAP II instructions to concentrations of approximately $1 \times 10^{10}$ PFU/ml and stored at 4°C.

**Genetic construction of *E. coli* OHHLux**

The OHHL specific bioluminescent bioreporter *E. coli* OHHLux was constructed by fusing the *luxCDABE* genes from *P. luminescens* with the *luxR* gene and *luxI* promoter region (P<sub>luxI</sub>) from *V. fischeri* into an EZ::TN pMOD cloning vector (Epicentre Technologies, Madison, WI) in conjunction with an *rrnB* T<sub>1</sub>T<sub>2</sub> transcriptional terminator and a kanamycin resistance gene (Fig. 6). The intermixing of the *P. luminescens* and *V. fischeri lux* genes was performed solely due to their ready availability in existing cloning vectors. The construct is similar to that of plasmid pSB401 created by Winson *et al.* (Winson *et al.* 1998), except our design incorporates the cloning region on a hyperactive transposon theoretically capable of insertion into virtually any bacterial chromosome.

The *rrnB* transcriptional terminator was PCR-amplified from pKK223-3 with the primer pairs 5′-ATCGATAAGAGTTTGTAGAAACGC-3′ and 5′-GAATTCTGTGGCAGGATG-3′ containing the restriction sites ClaI and *Eco*RI.
Figure 6. Cloning format for construction of an OHHL specific bioluminescent bioreporter *E. coli* OHHLux
(underlined) at the 5’ and 3’ ends, respectively and cloned into a pCR2.1-TOPO vector (Fig. 6A). The kanamycin gene was PCR-amplified from a pCR2.1-TOPO vector with the primer pairs 5´-AAGCTTTACGGGCGCAAGGCG-3´ and 5´-AAGCTTTACGGGCGCAAGGCG-3´ containing terminal HindIII restriction sites (underlined) and cloned into a pYES2.1/V5-His-TOPO vector (Invitrogen) (Fig. 6B). The luxCDABE gene cassette was PCR-amplified from P. luminescens using the primer pairs 5´-ATTAAATGGATGGCAAATAT-3´ and 5´-AGGATATCAACTATCAAAC-3´ and cloned into a pCR-XL-TOPO vector (Invitrogen) (Fig. 6C). The luxR gene and its neighboring P_luxI region were PCR-amplified from V. fischeri with the primer pairs 5´-GTCGACCCTCTATAGGTATAAAGCTTTACTTACGTG-3´ and 5´-GTCGACCCTCTATAGGTATAAAGCTTTACTTACGTG-3´ containing SalI restriction sites at both termini (underlined) and cloned into a pGEM-3Z vector (Promega) digested with SalI (Fig. 6D).

The rrnB TOPO clone was digested with ClaI and EcoRI and ligated into compatible ClaI and EcoRI sites within the MCS of the EZ::TN pMOD vector to create EZ::TN pMOD-rrnB (Fig. 6E). The kanamycin TOPO clone was digested with HindIII and ligated into the compatible HindIII site within the MCS of EZ::TN pMOD-rrnB to create EZ::TN pMOD-rrnB-Kn (Fig. 6F). The luxCDABE TOPO clone was digested with EcoRI (EcoRI sites are within the pCR-XL-TOPO MCS) and ligated into the compatible EcoRI site at the 3´ end of rrnB in the EZ::TN pMOD-rrnB-Kn vector to create EZ::TN pMOD-rrnB-luxCDABE-Kn (Fig. 6G). Directionality of the luxCDABE insert was confirmed by restriction digestion and sequencing. The luxR/P_luxI pGEM clone was digested with SalI and ligated into the SalI site of the MCS in EZ::TN pMOD-rrnB-
luxCDABE-Kn to create EZ::TN pMOD-rrnB-luxCDABE-luxR-Kn (Fig. 6H). Directionality of the luxR/P\textsubscript{lux} insert was confirmed by restriction digestion and sequencing. The original plan at this point was to remove the rrnB-luxCDABE-luxR-Kn sequence from the EZ::TN pMOD vector using existing flanking PshAI restriction sites to produce an EZ::TN transposon (Epicentre Technologies) for in vitro insertion into the chromosome of the host strain \textit{E. coli} XLOLR (Stratagene). However, transposition was not successful and the EZ::TN pMOD-rrnB-luxCDABE-luxR-Kn vector resides within \textit{E. coli} XLOLR as a plasmid.

**Dose response kinetics of the \textit{E. coli} OHHLux bioreporter to OHHL**

Synthetic OHHL (Sigma-Aldrich, St. Louis, MO; catalog no. K-3007) was diluted in 5 ml aliquots of M9 minimal media (Sambrook & Russell 2001) to desired concentrations and 100 μl of each dilution aliquot was added to triplicate wells in black 96-well microtiter plates (Dynex Technologies, Chantilly, VA). \textit{E. coli} OHHLux was grown in LB at 37°C to an OD\textsubscript{600} of 0.6 (~1 × 10\textsuperscript{8} CFU/ml) and 50 μl added to each microtiter plate well containing the diluted OHHL. As well, \textit{E. coli} OHHLux was added to wells void of OHHL to determine noninduced background levels of bioluminescence. Plates were sealed with transparent adhesive film (TopSeal-A, Perkin-Elmer, Boston, MA) and placed in a Perkin-Elmer Victor\textsuperscript{2} Multilabel counter at 30°C with shaking ('normal' speed was selected with a 0.5 mm orbital diameter) with light collection programmed for 1 s/well at 20 min intervals. In this and all experiments described below, resulting bioluminescent measurements were given the arbitrary light unit of counts/s (CPS). A bioluminescent response was considered significant if it achieved an intensity 2 standard deviations (2σ) above the negative control sample.
Specificity of the *E. coli* OHHLux bioreporter towards OHHL

Gram negative and Gram positive bacteria participate in quorum sensing communication networks via the production of many different types of AHL autoinducers or oligopeptides, respectively (Miller & Bassler 2001). To demonstrate that the *E. coli* OHHLux bioreporter responded to OHHL and not to other autoinducers, the bacteria listed in Table 5 were grown in media and at temperatures specified by the American Type Culture Collection (ATCC) to an OD$_{600}$ of 0.6 and individually combined 1:1 in 96-well microtiter plates with *E. coli* OHHLux grown to an OD$_{600}$ of 0.6 in LB at 37°C. Wells containing only *E. coli* OHHLux served as negative controls for monitoring background levels of bioluminescence. Positive control wells contained *E. coli* OHHLux and 10 nmol/l synthetic OHHL (final volume). Plates were incubated in the Victor$^2$ Multilabel counter at 30°C with shaking with bioluminescence monitored for 1 s/well at approximate 20 min intervals.

**Phage reporter pure culture assay**

To determine target cell detection limits, λ$_{luxI}$ reporter phage and *E. coli* OHHLux were combined with a dilution series of *E. coli* XL1-Blue down to an estimated 1 CFU/ml. λ$_{luxI}$ reporter phage were prepared on top agar overlays and stored at stock concentrations of $1 \times 10^{10}$ PFU/ml. The OHHLux bioluminescent bioreporter was grown in LB at 37°C to an OD$_{600}$ of 0.6 ($\sim 1 \times 10^8$ CFU/ml) and used as is. The *E. coli* host XL1-Blue was grown at 30°C in LB to an OD$_{600}$ of 0.7 ($\sim 1 \times 10^9$ CFU/ml) then serially diluted 1:10 down to approximately 1 CFU/ml in 50 ml conical centrifuge tubes containing 9 ml LB. One hundred microliter aliquots of each XL1-Blue dilution were
### Table 5. Strains tested for interference against the OHHLux bioluminescent bioreporter

<table>
<thead>
<tr>
<th>Organism</th>
<th>Autoinducer, oligopeptide, or autoinducer lactonase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrobacterium tumefaciens ATCC 33970</td>
<td>OOHL</td>
<td>(Fuqua &amp; Winans 1994)</td>
</tr>
<tr>
<td>Arthrobacter globiformis KACC 10580</td>
<td>AHL lactonase</td>
<td>(Park et al. 2003)</td>
</tr>
<tr>
<td>Bacillus mycoides ATCC 37015</td>
<td>AHL lactonase</td>
<td>(Dong et al. 2002)</td>
</tr>
<tr>
<td>Burkholderia cepacia ATCC 25416</td>
<td>OHL</td>
<td>(Lewenza et al. 1999)</td>
</tr>
<tr>
<td>Erwinia carotovora ATCC 15713</td>
<td>OHHL</td>
<td>(Pirhonen et al. 1993)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa ATCC BAA-47</td>
<td>OdDHL, BHL, AHL lactonase</td>
<td>(Huang et al. 2003)</td>
</tr>
<tr>
<td>Rhodobacter sphaeroides ATCC 55304</td>
<td>7,8-cis-N-(tetradecanoyl)-homoserine lactone</td>
<td>(Puskas et al. 1997)</td>
</tr>
<tr>
<td>Serratia liquefaciens ATCC 11367</td>
<td>HHL, BHL</td>
<td>(Eberl et al. 1996)</td>
</tr>
<tr>
<td>Staphylococcus aureus ATCC 35556</td>
<td>AIP (autoinducing peptide)</td>
<td>(Mayville et al. 1999)</td>
</tr>
<tr>
<td>Yersinia enterocolitica ATCC 23715</td>
<td>HHL, OHHL</td>
<td>(Throup et al. 1995)</td>
</tr>
</tbody>
</table>

OHHL, N-(3-oxohexanoyl)-homoserine lactone; OdDHL, N-(3-oxododecanoyl)-homoserine lactone; OHL, N-octanoyl-homoserine lactone; OOHL, N-(3-oxooctanoyl)-homoserine lactone; HHL, N-hexanoyl-homoserine lactone; BHL, N-butanoyl-homoserine lactone. For specifics on AHL lactonases and acylases, see Roche et al. (Roche et al. 2004).
then distributed columnwise (100 μl/well, 8 wells/column) throughout a black 96-well microtiter plate. A control column received 100 μl of LB/well. To all wells was then added 100 μl of λ<sub>luxI</sub> reporter phage stock (~1 × 10<sup>9</sup> PFU/ml final concentration) and 50 μl of OHHLux bioreporter (~5 × 10<sup>6</sup> CFU/ml final concentration). This equates to an approximate upper multiplicity of infection (MOI) of 10, and establishes a high infection rate of XL1-Blue cells. Plates were monitored for bioluminescence overnight in the Victor<sup>2</sup> Multilabel counter (30ºC, shaking, 1 s/well, 20 min intervals). Each XL1-Blue dilution tube was additionally incubated in a standard laboratory incubator with shaking (200 rev/min) at 37ºC to promote better growth than that achievable in the microtiter plate. After 5 h, 100 μl aliquots were removed from the preincubated dilution tubes as well as from a preincubated LB control tube and transferred to a microtiter plate as described above, with λ<sub>luxI</sub> reporter phage and OHHLux bioreporters added to each well also as described above. The microtiter plate was similarly monitored for bioluminescence.

All dilutions of XL1-Blue, both at the beginning and after the 5 h incubation period, were plated in triplicate on LB agar containing tetracycline at 14 mg/l (LB<sub>Tc</sub>) to determine viable cell counts. Each microtiter plate assay was also run with a duplicate control series of dilutions wherein <i>E. coli</i> XL1-Blue was replaced with the lambda resistant strain <i>E. coli</i> SOLR (Stratagene).

**Lettuce leaf wash assays**

<i>E. coli</i> XL1-Blue was grown at 30ºC in 200 ml LB to an OD<sub>600</sub> of 0.6 (~1 × 10<sup>8</sup> CFU/ml), centrifuged at 1000 × g for 10 min, and resuspended in 200 ml sterile water. A
A 1:10 dilution series was then prepared in 200 ml volumes of sterile water to form *E. coli* contaminated water ranging from $10^8$ to approximately 1 CFU/ml. A control tube not receiving an XL1-Blue inoculum was also prepared. Grocery store purchased iceberg head lettuce was rinsed with 1 l sterile water and spun dry in a kitchen salad spinner (Zyliss Corp., Foothill Ranch, CA). Ten grams of lettuce were placed in each dilution of *E. coli* contaminated water for 5 min with shaking (200 rev/min), spun dry in the salad spinner, and individually transferred to 30 ml of sterile saline. After 2 min of shaking (200 rev/min), saline diluents were transferred to 50 ml conical centrifuge tubes and centrifuged for 10 min at 3000 × g. Resulting pellets were resuspended in 3 ml LB and then assayed in microtiter plates either immediately or after a 16 h preincubation at 37ºC with shaking (200 rev/min). Preincubation was performed under tetracycline selection (14 mg/l final concentration) to select for XL1-Blue cells. Aliquots of 100 µl were removed from each LB resuspension either immediately or after the 16 h preincubation and transferred columnwise (100 µl/well, 8 wells/column, 1 column/dilution tube) to a 96-well black microtiter plate. Preincubated samples were washed once with LB to remove residual tetracycline just prior to sample transfer to the microtiter plate to prevent destruction of the OHHLux bioreporter. Each well then received 100 µl of λ*lux* reporter phage stock and 50 µl of OHHLux bioreporter prepared as described above for the pure culture assays. Microtiter plates were sealed with adhesive and monitored for bioluminescence in the Victor² Multilabel counter (30ºC, shaking, 1 s/well, 20 min intervals). Each LB resuspension was plated in triplicate on LBₜc plates both immediately and after the 16 h incubation to determine viable XL1-Blue cell counts.
Analytical measurement of OHHL

OHHL concentrations were analytically determined using a ThermoFinnigan LCQ DecaXPplus liquid chromatograph-mass spectrometer (LCMS) fitted with a 10 cm × 4.6 mm id C18 column (Advanced Chromatography Technologies, Chadds Ford, PA). Triplicate culture supernatant aliquots ranging from 1 to 50 ml were extracted twice with equal volumes of ethyl acetate, dried under nitrogen, and redissolved in 1 ml of methanol. A flow rate of 0.2 ml/min was used, starting with 20% methanol going to 95% methanol in 27 min with a 7 min hold, returning to 20% methanol in 6 min, and equilibrating for 5 min.

Results

OHHL dose response of the E. coli OHHLux bioreporter

The E. coli OHHLux bioreporter was exposed to varying concentrations of synthetic OHHL to determine detection limits (Fig. 7). Significant bioluminescent signals (2σ above background) were produced in response to OHHL at concentrations ranging from 10 nmol/l to 50 μmol/l. Saturation-type behavior was observed at OHHL concentrations exceeding 50 μmol/l. Since the function of this bioreporter is to detect low-level OHHL, we were more concerned with the lower tailed response curve profile. The inset to Figure 7 demonstrates response linearity at these lower OHHL concentrations ranging from 20 nmol/l to 2 μmol/l ($R^2 = 0.99$).
Synthetic OHHL was diluted at indicated concentrations and added in a 1:2 dilution to a culture of *E. coli* OHHLux, incubated for 1 h at 30°C, and then monitored for bioluminescence at 490 nm (*n* = 3). Inset shows linearity of the bioluminescent response within a lower detection range of 20 nmol/l to 2 μmol/l OHHL as measured by LCMS. Counts/s (CPS) were normalized to control CPS.
Specificity of the *E. coli* OHHLux bioreporter towards OHHL

The *E. coli* OHHLux bioreporter was co-cultured with bacterial strains synthesizing other classes of quorum sensing autoinducers or oligopeptides (Table 5). Significant bioluminescence was initiated only in response to the OHHL synthesizing strains *Erwinia carotovora* and *Yersinia enterocolitica*, which generated bioluminescence at 87% and 64%, respectively, that of control wells containing *E. coli* OHHLux exposed to 10 nmol/l synthetic OHHL (Fig. 8). The remaining strains produced bioluminescence at less than 1% of the *E. coli* OHHLux control.

Phage reporter assay in pure culture

To test assay detection limits and response times, a 1:10 dilution series of target *E. coli* XL1-Blue cells ranging from approximately $1 \times 10^8$ to 1 CFU/ml was added to λ_dlux reporter phage and *E. coli* OHHLux bioreporters in 96-well microtiter plates both with and without a supplementary 5 h preincubation. Without preincubation, the microtiter plate assay was capable of detecting target *E. coli* XL1-Blue cells at an initial cell concentration, as determined by plate counts, of $1.1 \times 10^8$ CFU/ml within 1.5 h, $2.0 \times 10^7$ CFU/ml within 2.2 h, $1.2 \times 10^6$ CFU/ml within 3.6 h, and $2.9 \times 10^5$ CFU/ml within 4.9 h (Fig. 9). XL1-Blue cell concentrations below $10^5$ CFU/ml did not generate significant bioluminescence. However, preincubating the dilution tubes at 37°C with shaking for 5 h prior to initiation of the assay permitted better growth of XL1-Blue cells than in the constrained microtiter plate wells, and allowed for detection down to 1 ($\pm 2.5$) CFU/ml within a total assay time of 10.3 h (Fig. 9). Duplicate control microtiter plates were also prepared substituting *E. coli* XL1-Blue with the lambda resistant strain *E. coli*.
*E. carotovora* and *Y. enterocolitica* synthesize OHHL and induced bioluminescence in the OHHLux bioreporter when co-cultured. Other quorum sensing autoinducers or oligopeptides produced by the remaining strains did not significantly induce bioluminescence from the OHHLux bioreporter (*n* = 3).

Figure 8. Bioluminescent response of *E. coli* OHHLux when co-cultured with each bacterium listed in Table 5.
Figure 9. Detection limits of the phage bioreporter assay in a pure culture of *E. coli* XL1-Blue target cells

*E. coli* XL1-Blue was diluted 1:10 from approximately $1 \times 10^8$ to 1 CFU/ml and combined with λ*lux* reporter phage and *E. coli* OHHLux bioreporters ($n = 3$). The assay directly detected *E. coli* XL1-Blue at mean concentrations ranging from $1.1 \times 10^8$ to $2.9 \times 10^5$ CFU/ml within 1.5 to 4.9 h, respectively. XL1-Blue cultures at concentrations below $10^5$ CFU/ml required a separate 5 h preincubation and yielded bioluminescent signals in as few as 1 (± 2.5) CFU/ml within 10.3 h, inclusive of the 5 h preincubation.
SOLR. No significant bioluminescence was observed in these plates (data not shown).

**Lettuce leaf rinse assays**

Rinsings from iceberg lettuce artificially contaminated with a 1:10 dilution series of *E. coli* XL1-Blue cells were exposed to the phage reporter assay. At the highest average concentration of XL1-Blue cells (1.4 × 10^8 CFU/ml), significant bioluminescence occurred within 2.6 h (Table 6). Successive 10-fold dilutions, yielding average cell concentrations of 1.5 × 10^7, 1.3 × 10^6, and 1.7 × 10^5 CFU/ml, generated significant bioluminescence within 3.3, 10.3, and 12.1 h, respectively. Cell concentrations below 10^5 CFU/ml did not produce significant bioluminescence. Therefore, these dilutions were preincubated under tetracycline selection for 16 h to increase target cell concentrations, and then tested in the phage reporter assay. After the 16 h preincubation, the control tube, void of an XL1-Blue inoculum, indicated a background concentration of nontarget tetracycline resistant cells of 3.6 × 10^8 CFU/ml. Estimated concentrations of tetracycline resistant XL1-Blue cells within comparable background populations were enumerated in each dilution tube based on similar colony morphology and are listed in Table 6. With selective overnight incubation, the original 10^4 and 10^3 inoculums of XL1-Blue cells could be detected within a total assay time, including the 16 h preincubation, of 19.1 h. The original 10^2 inoculum was detectable within 22.4 h. No significant bioluminescence was observed from XL1-Blue dilutions lower than 1 × 10^2 CFU/ml.
<table>
<thead>
<tr>
<th>Initial <em>E. coli</em> XL1-Blue inoculum (CFU/ml)</th>
<th>Estimated <em>E. coli</em> XL1-Blue concentration after 16 h pre-incubation (CFU/ml)</th>
<th>Time until bioluminescence induction (h)</th>
<th>Peak bioluminescence (CPS)</th>
</tr>
</thead>
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<tr>
<td>1.4 × 10^8</td>
<td>NA†</td>
<td>2.6</td>
<td>817,000</td>
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<td>1.5 × 10^7</td>
<td>NA</td>
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<td>1.7 × 10^5</td>
<td>NA</td>
<td>12.1</td>
<td>6,800</td>
</tr>
<tr>
<td>1.7 × 10^4</td>
<td>3.8 × 10^8</td>
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<td>81,800</td>
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<td>19.1‡</td>
<td>59,750</td>
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<td>20 (± 4)</td>
<td>9.6 × 10^4</td>
<td>ND§</td>
<td>ND</td>
</tr>
<tr>
<td>2 (± 3)</td>
<td>6.6 × 10^4</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Iceberg lettuce (10 g) was washed with water artificially contaminated at levels indicated
† NA, not assayed
‡ Includes a 16 h preincubation prior to initiation of the assay
§ ND, not detected
**Discussion**

The key technological metrics required by the food industry for effective detection and monitoring of bacterial pathogens are sensitivity, specificity, speed, simplicity, and cost-effectiveness. Portability can also be added to this list as quality control testing begins to move from the centralized laboratory to strategic on-the-spot monitoring within the production line itself. The traditional methods of selective sample enrichment followed by any number of morphological, biochemical, or serological tests offered accuracy and reliability but little in the way of rapidity, often requiring several days from initial sampling to final analysis. The introduction of nucleic acid based detection technologies (hybridization and PCR assays) afforded some significant increases in response times as well as improved sensitivity and specificity, but the complexity and costs involved in routine analysis limits their universal application. The binary phage assay described here provides a new direction towards pathogen monitoring. By exploiting phage specificity and the autoamplification power of quorum sensing, a single target pathogen can be manifested not as a single entity but rather as an intensified chemical signature. Detecting the chemical signature can then be accomplished using any variety of techniques ranging from highly sensitive but complex analytical methods (LCMS) to variously available easy-to-use AHL-sensing bioreporters (lacZ, gfp, or lux based) (Shaw et al. 1997, Camara et al. 1998, Winson et al. 1998, Andersen et al. 2001). We chose and constructed a luxCDABE based bioreporter (E. coli OHHLux) due to its capacity for autonomous, near real-time, and nondestructive sensing. Further, the ability to interface lux based bioreporters onto integrated circuit
microluminometers permits creation of miniaturized standalone biosensors capable of meeting the remote on-site monitoring needs of the food industry (Nivens et al. 2004).

*E. coli* OHHLux was designed to specifically respond to OHHL using the LuxI/LuxR quorum sensing regulatory system. Thus, infection of a host cell by a *luxI* recombinant phage leads to OHHL synthesis and subsequent bioluminescent signalling by the OHHLux bioreporter. However, bacteria naturally synthesizing OHHL will induce bioluminescence as well, as was shown in Figure 8 with *E. carotovora* and *Y. enterocolitica*. The presence of these and any other OHHL producing microorganisms in a sample would consequently generate false positive signals. It is therefore imperative that assays include a control consisting of bioreporter alone added to the sample to identify intrinsic OHHL.

*E. coli* OHHLux was assembled within an EZ::TN pMOD cloning vector (Epicentre Technologies) to permit chromosomal insertion. This provides advantages such as low reporter gene copy number for reduced background bioluminescence, genetic stability, and no requirements for antibiotic selection. However, chromosomal transposition was not successful and the pMOD-*lux* vector remained as a plasmid in *E. coli*. Unfortunately, this created an elevated but acceptable level of background bioluminescence and required initial growth under kanamycin selection, thereby creating an extra step in the assay procedure. We are currently working in conjunction with Epicentre Technologies to ascertain why transposition failed.

The binary phage reporter assay was developed using phage lambda as a model system for the general detection of *E. coli*. However, phage typing libraries, the host
groupings of over 5,000 bacteriophage, and the advent of phage therapy and phage anti-infectives now provide a considerable assemblage of phage from which to construct reporter systems with unique specificity towards select microbial pathogens (Ackerman 2001, Stone 2002). For example, phage PP01 can be used to specifically detect \( E. coli \) O157:H7, even within a background of \( E. coli \) strains of other O-serogroups and K-12 strains (Morita et al. 2002b). Although the emergence of phage resistance in target hosts is often cited as a disadvantage in phage based detection strategies, the use of multi-phage cocktails and the natural reversion of host mutants back to phage sensitivity often negates this perceived obstacle (O'Flynn et al. 2004).

The reporter phage assay was capable of direct, rapid (1.5 – 5 h) detection of \( E. coli \) XL1-Blue in pure culture at high cell densities (\( 10^8 – 10^5 \) CFU/ml) using a three-step protocol consisting of the addition of reporter phage, bioreporter, and target cells (Fig. 9). No other reagents or manipulations were required other than incubation and light measurement, making this assay highly amenable to automated high throughput monitoring using any number of available photomultiplier based luminescent readers. Phage based sensing also ensures only the detection of live cells, thereby overcoming the false positive detection of dead cells often inherent in other assaying methods such as ELISA and PCR (Wolffs et al. 2005).

Detection of \( E. coli \) XL1-Blue in pure culture at cell densities below \( 10^5 \) CFU/ml could not be achieved directly within the microtiter plate assay. Adequate growth of XL1-Blue cells at these lower cell densities was not possible due to a combination of several factors (i.e., competition for growth resources with the OHHLux bioreporter cell population, coexistence with the \( \lambda_{lux} \) reporter phage population, and a poorly oxygenated
environment within the sealed microtiter plate well). It was therefore necessary to preincubate XL1-Blue cells separately in well-aerated test tubes for 5 h to increase initial cell numbers prior to initiation of the phage reporter assay. This permitted detection at an initial cell concentration of 1 (± 2.5) CFU/ml within a total assay time of 10.3 h (Fig. 9).

When tested against artificially contaminated lettuce, the assay detected original inoculums between $10^8 - 10^5$ CFU/ml within 2.6 – 12.1 h, respectively (Table 6). This occurred without specific selection for target XL1-Blue cells or additional preincubation steps outside of the normal microtiter plate assay. In these experiments *E. coli* XL1-Blue was detected within a background of normal microflora, therefore detection times were longer than in the pure culture experiments. This would be expected since contaminating cells nonspecifically bind with phage, thereby reducing effective phage concentrations. (Similar nonspecific binding would occur in food samples heavily endowed with particulate matter, such as ground beef. Preprocessing of such samples to remove particulates would likely be necessary.) This also caused decreased levels of bioluminescence; as phage titers decline so do phage infection events, and the corresponding reduction in OHHL synthesis resulted in lowered induction rates of the OHHLux bioreporter. Additionally, growth of XL1-Blue populations was slower due to competition from other cell populations. Collectively, these factors make quantitative assessment of target bacteria difficult, making this assay more of a sentinel first response monitoring system capable of presence/absence detection to be followed with more robust analytical testing.

As with the pure culture assays, detection below $10^5$ XL1-Blue cells/ml was not possible without prior incubation. As well, tetracycline was used to select for XL1-Blue
and reduce nontarget background microbial populations. When assaying for an actual pathogen, relevant enrichment methods besides mere antibiotic addition are available and would have to be chosen based on the phage/pathogen combination being used (Sharpe 2001). Although preincubations were carried out under selective conditions, the background concentration of nontarget cells remained high \((3.6 \times 10^8 \text{ CFU/ml})\). This prevented optimal growth of \(E. coli\) XL1-Blue populations and once more served as a sink for nonspecific phage attachment. As a result, detection times were again higher than those seen with pure culture assays with corresponding XL1-Blue concentrations. In addition, light levels decreased, as observed in Table 6 when comparing, for example, initial inoculums of \(1.4 \times 10^8 \text{ CFU/ml}\) versus \(1.7 \times 10^4 \text{ CFU/ml}\). Although with preincubation the \(1.7 \times 10^4 \text{ CFU/ml}\) culture eventually grew to an estimated \(3.8 \times 10^8 \text{ CFU/ml}\), the presence of a large nontarget microbial population and corresponding reduction in phage titer due to nonspecific binding resulted in bioluminescence levels 10-fold lower than that of the \(1.4 \times 10^8 \text{ CFU/ml}\) culture (817,000 CPS versus 81,800 CPS). Detection of XL1-Blue at and below 10 CFU/ml failed due to insufficient enrichment to detectable numbers during the preincubation.

The preliminary testing reported here demonstrates the unique ability of \(luxI\)-incorporated bacteriophage to detect target microbes within a simple and cost-effective microtiter plate format. Although detection limits do not yet approach that of more routine monitoring methods such as PCR and ELISA, ongoing genetic enhancements to the reporter phage (i.e., expression of multiple phage based \(luxI\) elements rather than a single element) as well as better bacterial target enrichment and isolation techniques (i.e.,
immunomagnetic separation coupled with phage based sensing (Goodridge et al. 1999, Favrin et al. 2001)), may drive detection limits to acceptable levels.
Chapter III: Bacteriophage based bioluminescent bioreporter

for the detection of *E. coli* O157:H7
This is a version of an article found in the journal of Food Pathogen Detection entitled “Bacteriophage Based Bioluminescent Bioreporter for the Detection of *Escherichia coli* O157:H7”.

Abstract

Rapid detection of pathogenic bacteria in food and water is vital for the prevention of foodborne illness. In this work, the *lux* reporter genes were used in a new bioassay that allows for pathogen monitoring without multiple sample manipulations or addition of exogenous substrate. A recombinant phage specific for *Escherichia coli* O157:H7 was constructed that, upon infection, catalyzes the synthesis of *N*-(3-oxohexanoyl)-*l*-homoserine lactone (OHHL). This phage PP01 derivative carries the *lux* gene from *Vibrio fischeri* under the control of the phage promoter P <sub>L</sub>. OHHL produced by infected *E. coli* O157:H7 induces bioluminescence in bioreporter cells carrying the *V. fischeri lux* operon. The ability of phage PP01-*lux* to detect *E. coli* O157:H7 was confirmed in a 96-well plate assay. In this assay, *luxCDABE* bioreporter cells capable of detecting OHHL were mixed with phage PP01-*lux* and *E. coli* O157:H7, and luminescence was monitored. Reporter phage induced light in bioreporter cells within 2 h when exposed to 10<sup>4</sup> CFU/ml *E. coli* O157:H7, and were able to detect 10 CFU/ml in pure culture with a pre-incubation step (total detection time 5 h). The detection method was also applied to contaminated apple juice and was able to detect 10<sup>4</sup> CFU/ml in 2 h after a 6 h pre-incubation.
Introduction

Rapid detection methods for *E. coli* O157:H7 in food and water are necessary to detect both naturally occurring outbreaks and bioterrorist events. A variety of methods exist for detection of *E. coli* O157:H7, but most are slow or require significant sample manipulation (Moxley 2003, Deisingh & Thompson 2004, Gehring et al. 2004, Lee et al. 2005). Culture methods require skilled personnel and results are not obtained for 24 - 48 h (Moxley 2003, Deisingh & Thompson 2004). ELISAs and other immunological methods require staining and washing steps (Moxley 2003, Deisingh & Thompson 2004, Gehring et al. 2004). Rapid detection methods based on PCR are fast and reliable but cannot differentiate between live and dead bacterial cells and can be fairly complex (Deisingh & Thompson 2004). Biosensor based systems can be rapid and amenable to real-time monitoring, but currently cannot detect low concentrations of bacteria, and have difficulty detecting bacteria in food matrices (Radke & Alocilja 2005, Subramamanian et al. 2006).

The specificity of bacteriophage for their bacterial hosts has long been used for the identification of bacterial pathogens. Phage lysis assays are commonly used for identification and typing of bacteria (reviewed in (Marks 2000)). In the last 20 years, genetic manipulation of bacteriophage to make “reporter phage” using reporter genes such as bacterial luciferase (*luxAB*), β-galactosidase (*lacZ*) and green fluorescent protein (*gfp*) has been documented (Ulitzer & Kuhn 1987, Stewart et al. 1989, Kodikara et al. 1991, Turpin et al. 1993, Chen & Griffiths 1996, Loessner et al. 1996, Waddell & Poppe 2000, Goodridge & Griffiths 2002). Phage are metabolically inert when outside of their host, so the reporter proteins encoded in these phage are not produced until the
appropriate bacterium is infected. In the existing lux based phage reporter systems, bacteria are detected following phage infection, an incubation/growth period, and the addition of an exogenous substrate (Kodikara et al. 1991, Chen & Griffiths 1996, Loessner et al. 1996). While these methods are capable of specific and sensitive detection of bacteria, they are not amenable to continuous monitoring because they require excessive sample manipulation.

Recently, a rapid GFP-reporter phage detection system was described that can detect and differentiate live and dead E. coli O157:H7 cells (Oda et al. 2004, Awais et al. 2006). This GFP reporter was constructed in bacteriophage PP01, which was isolated from swine stools by Morita et al. (Morita et al. 2002b). Phage PP01 was found to infect a variety of E. coli O157:H7 strains (17 out of 19 tested, including toxigenic and non-toxigenic), but none of the 14 non-O157:H7 E. coli strains examined (Morita et al. 2002b, O'Flynn et al. 2004). Other species of bacteria that have been tested, including Salmonella enterica serovar Typhimurium, Pseudomonas fluorescens, Pseudomonas aeruginosa, Citrobacter freundii, Erwinia carotovora, and Yersinia enterocolitica, have not shown susceptibility to the phage, (Morita et al. 2002b, O'Flynn et al. 2004). This PP01-GFP reporter phage is rapid and specific, but like all GFP-based assays it requires a high energy light source for excitation of GFP which may limit its potential for use in real-time online industrial and environmental monitoring. Here, we describe a PP01 reporter phage that uses the lux reporter genes in a unique construct that permits detection without the addition of substrate, and has potential for use in real-time online monitoring.

The lux bioluminescence genes were isolated from the marine bacterium Vibrio fischeri, and includes a cassette of five genes (luxCDABE) whose products are necessary
for the generation of light, and two genes (\textit{luxI} and \textit{luxR}) that produce the components of a quorum sensing system that activates the transcription of the \textit{luxCDABE} cassette (Meighen 1991). The luciferase genes (\textit{luxAB}) encode the proteins responsible for generating bioluminescence, while the reductase (\textit{luxC}), transferase (\textit{luxD}) and synthetase (\textit{luxE}) genes encode proteins that produce an aldehyde substrate required for the bioluminescence reaction. Bioluminescent bacteriophage based bioreporters developed in the past carry the \textit{luxAB} genes, but lack the \textit{luxCDE} genes (Ulitzer & Kuhn 1987, Stewart et al. 1989, Kodikara et al. 1991, Chen & Griffiths 1996, Loessner et al. 1996, Waddell & Poppe 2000). Thus, once these bacteriophage infect their target cells, detection requires the addition of exogenous substrate. The entire \textit{luxCDABE} cassette is too large to be accommodated by most phage genomes, so to eliminate the need for exogenous substrate an alternative approach is necessary.

The detection system described here takes advantage of the \textit{luxI}/\textit{luxR} quorum sensing system. Quorum sensing systems are cell-to-cell communication networks based on the synthesis of diffusible autoinducer molecules (reviewed in (Miller & Bassler 2001)). \textit{V. fischeri} expresses low levels of \textit{luxI} and produces small amounts of the diffusible molecule \textit{N-(3-oxohexanoyl)-L-homoserine lactone} (OHHL). When a high concentration of \textit{V. fischeri} is present the concentration of OHHL becomes high enough to bind to LuxR and activate transcription of \textit{luxCDABE} and \textit{luxI}, causing the production of light. In the system used here, the complete \textit{lux} cassette (\textit{luxCDABE}) along with the transcriptional regulator \textit{luxR} are carried by a “bioreporter cell” that detects the diffusible OHHL produced by a target pathogen infected with a phage carrying the \textit{luxI} gene (Fig. 10). The initial feasibility of such phage based bioreporters was demonstrated with a
phage lambda/E. coli K12 model (Ripp 2006). In this work, luxI was integrated into phage PP01 for detection of its host, E. coli O157:H7, in pure culture and in artificially contaminated apple juice.

Materials and methods

Bacterial strains and bacteriophage

Bacteriophage PP01 was obtained from Hajime Unno (Tokyo Institute of Technology), and was stored in SM buffer (10 mM MgSO$_4$, 100 mM NaCl, 0.01% gelatin, and 50 mM Tris-HCl, pH 7.5). The bacterial strains used in this work were obtained from ATCC: toxigenic E. coli O157:H7 (43895), non-toxigenic E. coli O157:H7 (43888), E. coli K12 (10798), E. coli DH1 (33849), E. coli MM294 (33625), Bacillus cereus (14579) and Bacillus mycoides (6462). All strains were grown in Luria Bertani medium (LB; 10 g tryptone, 5 g yeast extract, 10 g NaCl per liter H$_2$O, pH 7.0). The bioreporter used in this work was E. coli OHHLux which has been previously described (Ripp 2006). Briefly, the bioreporter cells carry luxR and luxCDABE under the control of the bidirectional lux promoter. Activation of the lux operon in these cells occurs in response to OHHL at concentrations of 5 nM and higher.

Construction of the reporter phage

The methods used for the construction of the PP01-luxI reporter phage were based on those used by Oda et al. (Oda et al. 2004) to create a PP01-GFP phage. Construction of the luxI-carrying plasmid used for recombination of PP01 entailed the fusion of the V.
Figure 10. Bacteriophage based bioluminescent bioreporter for pathogen detection

Bacteriophage based bioluminescent bioreporter for pathogen detection. Phage PP01-\textit{luxI}, which carries the \textit{luxI} gene under the control of a strong phage promoter, injects its genome into \textit{E. coli} O157:H7. Upon infection, \textit{luxI} is transcribed from the phage genome and as a result N-(3-oxohexanoyl)-L-homoserine lactone (OHHL) is produced. OHHL diffuses out of the host cell and into a nearby bioreporter cell where it binds to the transcriptional activator LuxR. The LuxR-OHHL complex interacts with P\textsubscript{L} to activate transcription of \textit{luxCDABE} resulting in the production of a detectable light signal.
The *luxI* gene was PCR-amplified from *V. fischeri* using forward primer 5'-
\textbf{CATATGACTATAATGATAAAAAAATCGG-}3' and reverse primer 5'-
\textbf{CATATGTTAAATTAAGACTGC-}3' to introduce the unique restriction site *NdeI* at both
termini (underlined) and cloned into a pCR2.1-TOPO vector (Fig. 11A). The *luxI* gene
was excised from the TOPO vector by *NdeI* restriction digestion and ligated into the *NdeI*
multicloning site (MCS) of the pLEX vector, thereby placing *luxI* in frame with the
Lambda P_{L} promoter (Fig. 11B). Directionality was confirmed by restriction digestion
and sequencing.
The *rrnB* transcriptional terminator was PCR-amplified from pKK223-3 using forward primer 5'-ATCGATAAAGAGTTTTGTAGAAACGC-3' and reverse primer 5'-CTGGTTTTGGCGGATG-3' to introduce the restriction site *Cla*I at the 5' end (underlined) and cloned into a pCR2.1-TOPO vector (Fig. 11C).

The *P_L-luxI* fusion was PCR-amplified from pLEX with forward primer 5'-ATCGATGTGACCTAGAGAGGATCC-3' and reverse primer 5'-ATCGATTTCAGGCTTCTCTGACCATA-3' containing the restriction sites *Cla*I (underlined) and cloned into a pCR2.1-TOPO vector (Fig. 11D). This vector was then digested with *Cla*I and ligated into the *Cla*I site of the *rrnB* TOPO vector described above to create a *P_L-luxI-rrnB* fusion within a pCR2.1-TOPO vector (Fig. 11E). Directionality was again confirmed by restriction digestion and sequencing.

The *soc2soc1* genes of phage PP01 were amplified using forward primer 5'-GCGTCGACGAAGAAATCTTTAAACTTTATCTCTG-3' and reverse primer 5'-CAGTCGACTCTCTCTTTATTTAAATTACATGAC-3' to introduce *Sal*I restriction sites (underlined) and cloned into a pCR2.1-TOPO vector (Fig. 11F). The *soc2soc1* genes were excised from the TOPO vector by digestion with *Sal*I and ligated into the *Sal*I MCS of pCR2.1-*P_L-luxI-rrnB* just upstream of *P_L* (Fig. 11G). Directionality was confirmed by restriction digestion and sequencing.

The *soc* gene of phage PP01 was amplified using forward primer 5'-AAGATATCCATGGCTAGTACTCGCGGTAA-3' and reverse primer 5'-TCTCTCGAGGGTTAATCCAAAGCTTAAACAT-3' to introduce *Eco*RV and *Xho*I restriction sites, respectively (underlined) and inserted into the TA cloning vector
Figure 11. Genetic construction of plasmid pCR2.1-soc2soc1-P_L-luxI-rrnB-soc
pCR4.0-TOPO vector (Fig. 11H). The soc gene was excised from the TOPO vector by digestion with EcoRV and XhoI, and ligated into the EcoRV/XhoI MCS of pCR2.1-soc2soc1-P_L-luxI-rrnB just downstream of rrnB (Fig. 11I). Insertion was confirmed by restriction digestion and sequencing.

The completed construct pCR2.1-soc2soc1-P_L-luxI-rrnB-soc was electroporated into E. coli O157:H7 (ATCC 43888, non-toxigenic) and transformed cells were selected on LB plates containing 50 µg Kanamycin/ml. Log phase cultures of E. coli O157:H7 containing pCR2.1-soc2soc1-P_L-luxI-rrnB-soc were infected with phage PP01 at a multiplicity of infection of 0.01 and 0.1 and incubated 5 h at 37°C to allow homologous recombination between the plasmid and the phage to occur (Fig. 12). Bacteria were lysed by addition of chloroform followed by a 15 min incubation at 37°C. Cell debris was pelleted, and phage were titered in top agar overlays.

Recombinant PP01-luxI phage were detected with a plaque hybridization assay using the Alkphos Direct Labeling and Detection System (Amersham Biosciences). A probe for luxI was obtained by amplifying a 1 kb portion of the luxI gene using the forward primer 5'-GGATCCAAAAATACACTTGACCATT-3' and the reverse primer 5'-GAGCTCTTATTTGATGCCTGGCAGTT-3', and labeled according to the kit directions. Hybridization was performed according to kit directions. Labeled plaques were visualized with a Molecular Dynamics Storm 840 phosphorescent imager. Phage that were labeled in the hybridization assay were propagated, and the presence of luxI in these phage was confirmed via PCR, using the forward primer 5'-GCAATTCCATCGGAGGAGTA-3' and the reverse primer 5'-
Figure 12. Homologous recombination between plasmid pCR2.1-soc2soc1-P_L-luxI-rrnB-soc and phage PP01

Crossover events occur in the soc2soc1 and soc regions, resulting in the integration of P_L-luxI-rrnB into the phage PP01 genome.
GCACTCTGTGGACCAAGCAA-3’. One confirmed PP01-*luxI* phage clone was propagated and used for all subsequent experiments.

**Detection of *E. coli* O157:H7 in pure culture**

To detect *E. coli* O157:H7 in pure culture, PP01-*luxI* reporter phage and *E. coli* OHHLux bioreporter cells were combined with a dilution series of *E. coli* O157:H7. Phage PP01-*luxI* was diluted to a concentration of $10^8$ PFU/ml in SM buffer. The *E. coli* OHHLux bioreporter cells were grown overnight in LB at 28°C to an OD$_{600}$ of 0.35 (~$1 \times 10^8$ CFU/ml). The volume and concentration of *E. coli* OHHLux used for detection of OHHL in the phage bioreporter system was previously optimized (unpublished data).

Host *E. coli* O157:H7 (ATCC 43888) was grown in LB at 37°C to an OD$_{600}$ of 0.2 (~$1 \times 10^8$ CFU/ml), then serially diluted 1:10 down to 10 CFU/ml in 9 ml LB in 50 ml conical tubes. Samples of each *E. coli* O157:H7 dilution were then distributed into a black 96-well microtiter plate (Dynex Technologies, Chantilly, VA) (100 µl/well). Next, PP01-*luxI* reporter phage (100 µl/well, ~$1 \times 10^7$ PFU/ml final concentration) and *E. coli* OHHLux bioreporter cells (100 µl/well, ~$1 \times 10^7$ CFU/ml final concentration) were added to all wells. Control wells included *E. coli* OHHLux bioreporter cells (100 µl/well) alone and mixed with LB (100 µl/well), *E. coli* O157:H7 (100 µl/well, ~$1 \times 10^8$ CFU/ml) and PP01-*luxI* (100 µl/well) in separate wells. The plate was sealed with a Breathe-Easy (Diversified Biotech, Boston, MA) membrane, and monitored for bioluminescence every 30 min over a 20 h period in a Perkin-Elmer 1450 Microbeta Plus liquid scintillation counter at 30°C.
To increase the sensitivity of this assay, a pre-incubation step was added. Each tube of the *E. coli* O157:H7 dilution series was additionally incubated with shaking (200 rev/min) for 3 h at 37°C to promote growth. After 3 h, 100 µl samples were removed from the pre-incubated dilution tubes and transferred to a microtiter plate as described above, with phage PP01-*luxI* and *E. coli OHHLux* bioreporters added to each well also as described above. The microtiter plate was sealed and monitored for bioluminescence as described above. A student t-test was performed to determine the point at which luminescence in test wells significantly exceeded luminescence from control wells (P < 0.05).

**Specificity of assay for *E. coli* O157:H7**

The specificity of phage PP01 for *E. coli* O157:H7 is well established (Morita et al. 2002a, Morita et al. 2002b, O’Flynn et al. 2004), but the specificity of the recombinant PP01-*luxI* phage needed to be confirmed experimentally. More importantly, the ability of phage PP01-*luxI* to infect a toxigenic *E. coli* O157:H7 strain required experimental confirmation. To accomplish this, toxigenic *E. coli* O157:H7 (43895), along with several control strains (*E. coli* K12, *E. coli* DH1, *E. coli* MM294, *B. cereus* and *B. mycoides*) were tested in the reporter assay. The toxigenic strain of *E. coli* O157:H7 (43895) was grown in LB to an OD$_{600}$ of 0.2 (~1 × 10$^8$ CFU/ml), then serially diluted 1:10 down to 1 × 10$^5$ CFU/ml in LB. Control strains (*E. coli* K12, *E. coli* DH1, *E. coli* MM294, *B. cereus* and *B. mycoides*) and the known host strain *E. coli* O157:H7 (43888) were grown at 37°C to concentrations of ~10$^8$ CFU/ml. Phage PP01-*luxI* was diluted to a concentration of 10$^8$ PFU/ml in SM buffer. The *E. coli OHHLux* bioreporter cells were grown in LB at 28°C to an OD$_{600}$ of 0.35. One hundred microliter samples of each *E. coli* O157:H7
dilution and each control strain were then distributed into a black 96-well microtiter plate (100 µl/well). Next, 100 µl of PP01-\textit{luxI} reporter phage (~1 × 10⁷ PFU/ml final concentration) and 100 µl \textit{E. coli} OHHLux bioreporter cells (~1 × 10⁷ CFU/ml final concentration) were added to all wells. Controls were as described above. The plate was sealed and monitored for bioluminescence as described above.

**Detection of \textit{E. coli} O157:H7 in apple juice**

Phage PP01-\textit{luxI} was diluted to a concentration of 10⁸ PFU/ml in SM buffer. The \textit{E. coli} OHHLux bioreporter cells were grown in LB at 28°C to an OD₆₀₀ of 0.35. Host \textit{E. coli} O157:H7 (ATCC 43888) was grown at 37°C in LB to an OD₆₀₀ of 0.2 (~1 × 10⁸ CFU/ml). The cells were pelleted by centrifugation (10 min at 3000 × g), and suspended in an equal volume of commercial pasteurized apple juice. A 1:10 dilution series of this culture was made in apple juice down to ~1 CFU/ml. For detection, 0.5 ml of the artificially contaminated apple juice was added to 3 ml of LB in a culture tube and pre-incubated in a 40°C shaker-incubator for 1 to 6 h prior to detection. Pure apple juice (0.5 ml) was mixed with 3 ml LB and incubated overnight as a control. One hundred microliter samples of each \textit{E. coli} O157:H7 dilution were then distributed into a black 96-well microtiter plate (100 µl/well). Next, 100 µl of PP01-\textit{luxI} reporter phage (~1 × 10⁷ PFU/ml final concentration) and 100 µl \textit{E. coli} OHHLux bioreporter cells (~1 × 10⁷ CFU/ml final concentration) were added to all wells. Control wells included \textit{E. coli} OHHLux bioreporter cells (100 µl/well) alone and mixed with the LB/apple juice mixture (100 µl/well), \textit{E. coli} O157:H7 (100 µl/well, ~1 × 10⁸ CFU/ml) and PP01-\textit{luxI} (100 µl/well) in separate wells. The plate was sealed and monitored for bioluminescence as described above.
Results

PP01-luxI reporter phage assay in pure culture

The ability of phage PP01-luxI to detect *E. coli* O157:H7 at concentrations ranging from $10^8$ CFU/ml to 10 CFU/ml in LB was examined. Phage PP01-luxI was mixed with *E. coli* OHHLux bioreporter cells and *E. coli* O157:H7 (ATCC 43888, a non-toxigenic strain) in a 96-well plate and monitored for bioluminescence emission. At a concentrations of $10^4$ - $10^8$ CFU/ml, the assay could rapidly (<2 h) detect *E. coli* O157:H7 without a pre-incubation step (Fig. 13). To lower the minimum level of detection, *E. coli* O157:H7 samples were pre-incubated for 3 h at 37°C prior to the assay, which resulted in a detection limit of ~10 CFU/ml (initial concentration) in a total time (pre-incubation and assay) of approximately 4 h (Table 7).

Specificity of PP01-luxI reporter phage assay

The specificity of phage PP01 for *E. coli* O157:H7 is well established (Morita et al. 2002a, Morita et al. 2002b, O'Flynn et al. 2004), and the specificity of the OHHLux bioreporter cells used in this work has been previously described (Ripp 2006). To demonstrate that our assay system did not deviate from the specificities of its components, its ability to detect a toxigenic strain of *E. coli* O157:H7 (ATCC 43895) was confirmed at concentrations of $10^5$ – $10^8$ CFU/ml. It was also shown to not produce significant light in response to several non-O157:H7 (*E. coli* K12, *E. coli* DH1, *E. coli* MM294) and non-*E. coli* (*B. cereus, B. mycoides*) bacterial strains at concentrations of $10^8$ CFU/ml (Fig. 14). *B. cereus* and *B. mycoides* were tested because these bacteria are
Figure 13. Detection of *E. coli* O157:H7 without pre-incubation

*E. coli* OHHLux bioreporters ($10^8$ CFU/ml, 100 µl/well) were mixed with *E. coli* O157:H7 ($10^4$ - $10^8$ CFU/ml, 100 µl/well) and phage PP01-*luxI* ($10^8$ PFU/ml, 100 µl/well) in the wells of a 96 well plate (labeled as $10^8$ (♦), $10^7$ (■), $10^6$ (▲), $10^5$ (◇), and $10^4$ (□)). Control wells contained *E. coli* OHHLux bioreporters alone or mixed with LB, *E. coli* O157:H7 ($10^8$ CFU/ml) or phage PP01-*luxI* (labeled as OHHLux (×), OHHLux + LB (△), OHHLux + $10^8$ (○), and OHHLux + phage (●), respectively). Light production was monitored, and is shown as luminescent counts per second (LCPS). Error bars represent the standard deviation of six replicate wells.
### Table 7. LCPS in pure culture averaged across all five *E. coli* O157:H7 strains tested

<table>
<thead>
<tr>
<th>Initial concentration of <em>E. coli</em> O157:H7 (CFU/ml)</th>
<th>Pre-incubation time (h)</th>
<th>Total assay time including pre-incubation (h)</th>
<th>LCPS at time of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^8$</td>
<td>0</td>
<td>0.5</td>
<td>$6,993 \pm 130$</td>
</tr>
<tr>
<td>$10^7$</td>
<td>0</td>
<td>1.0</td>
<td>$16,889 \pm 2,065$</td>
</tr>
<tr>
<td>$10^6$</td>
<td>0</td>
<td>1.0</td>
<td>$11,914 \pm 718$</td>
</tr>
<tr>
<td>$10^5$</td>
<td>0</td>
<td>1.0</td>
<td>$11,417 \pm 627$</td>
</tr>
<tr>
<td>$10^4$</td>
<td>0</td>
<td>1.0</td>
<td>$11,255 \pm 599$</td>
</tr>
<tr>
<td>$10^3$</td>
<td>3</td>
<td>4.0</td>
<td>$14,011 \pm 412$</td>
</tr>
<tr>
<td>$10^2$</td>
<td>3</td>
<td>4.0</td>
<td>$13,882 \pm 357$</td>
</tr>
<tr>
<td>10</td>
<td>3</td>
<td>4.0</td>
<td>$13,796 \pm 463$</td>
</tr>
<tr>
<td>0 (control)</td>
<td>n/a</td>
<td>0.5 – 4.0</td>
<td>$3,771 \pm 763$ –</td>
</tr>
</tbody>
</table>

$4,286 \pm 119$

*a* Data shown for first reading in which the averaged luminescent counts per second (LCPS) of the contaminated samples significantly exceeded the LCPS of the controls. Background luminescence slowly increases over time. Control values shown are from the control wells giving the highest luminescence at the time of detection.
commonly found in the environment but the inability of phage PP01 to infect these bacteria was not previously demonstrated.

**PP01-luxI reporter phage assay to detect *E. coli* O157:H7 in apple juice**

Outbreaks of *E. coli* O157:H7 infection have been linked to products such as unpasteurized apple juice (CDC 1996b, Cody et al. 1999). The ability to detect *E. coli* O157:H7 in apple juice was tested as a potential application for the PP01-*luxI* phage detection system. Apple juice was artificially contaminated with *E. coli* O157:H7 at concentrations from approximately 1 CFU/ml to $10^8$ CFU/ml. An initial experiment revealed that detection in undiluted apple juice was not possible, as no light was produced even at the highest concentration of bacteria, even if the samples were brought to a neutral pH (data not shown). To circumvent this problem, samples were diluted into LB (0.5 ml inoculated apple juice to 3 ml LB) and grown at 40°C for 1 to 6 h prior to detection. The higher incubation temperature was used to favor growth of *E. coli* O157:H7 over background microflora (Vimont et al. 2006). This method allowed detection of *E. coli* O157:H7 at $10^8$ CFU/ml in under 3 h (1 h preincubation, 1.5 h assay), and down to $10^4$ CFU/ml in under 8 h (6 h preincubation, 1.5 h assay) (Table 8). No significant bioluminescence was observed when *E. coli* concentrations were below $10^4$ CFU/ml.

**Discussion**

*E. coli* O157:H7 is an ongoing threat because of naturally occurring foodborne outbreaks and its potential for use in bioterrorism. Rapid methods for detection of *E. coli*
Figure 14. Cross reactivity of the PP01-\textit{luxI} detection system

Potential host cells (10^8 CFU/ml, 100 µl/well) were mixed with phage PP01-\textit{luxI} (10^8 PFU/ml, 100 µl/well) and \textit{E. coli} OHHLux bioreporter cells (10^8 CFU/ml, 100 µl/well) in the wells of a 96 well plate. Light production was monitored, and peak luminescence is shown as luminescent counts per second (LCPS). Data were normalized by subtracting the background luminescence of \textit{E. coli} OHHLux bioreporter cells. Error bars represent the standard deviation of three replicate wells.
Table 8. Detection of *E. coli* O157:H7 ATCC 43888 in apple juice

<table>
<thead>
<tr>
<th>Initial concentration of <em>E. coli</em> O157:H7 (CFU/ml)</th>
<th>Pre-incubation time (h)</th>
<th>Total assay time including pre-incubation (h)</th>
<th>LCPS at time of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^8$</td>
<td>1</td>
<td>2.4</td>
<td>$125,110 \pm 12,448$</td>
</tr>
<tr>
<td>$10^7$</td>
<td>2</td>
<td>4.5</td>
<td>$203,026 \pm 16,778$</td>
</tr>
<tr>
<td>$10^6$</td>
<td>4</td>
<td>5.9</td>
<td>$152,606 \pm 14,281$</td>
</tr>
<tr>
<td>$10^5$</td>
<td>5</td>
<td>5.9</td>
<td>$337,780 \pm 22,202$</td>
</tr>
<tr>
<td>$10^4$</td>
<td>6</td>
<td>7.6</td>
<td>$236,900 \pm 64,701$</td>
</tr>
<tr>
<td>0 (control)</td>
<td>n/a</td>
<td>2.4 – 7.6</td>
<td>$12,836 \pm 1739 – 44,843 \pm 10,861$</td>
</tr>
</tbody>
</table>

aData shown for first reading in which the luminescent counts per second (LCPS) of the contaminated sample significantly exceeded the LCPS of the controls. Background luminescence slowly increases over time. Control values shown are from the control wells giving the highest luminescence at the time of detection.
O157:H7 in the product production line and in drinking and recreational water sources are needed to minimize the human health impact of this pathogen. While there are a wide variety of assays available for the detection of *E. coli* O157:H7, very few are amenable to adaptation for real-time use in production line and environmental monitoring. The GFP-PP01 phage assay introduced by Oda et al. (Oda et al. 2004) was a step toward this, but the required light source to excite GFP restricts its potential for use in miniaturized remote sensing devices. Detection methods based on the bioluminescent *lux* system can easily be integrated into an existing technology, the bioluminescent bioreporter integrated circuit (BBIC) (Nivens et al. 2004), for use in real-time remote monitoring.

As a first step toward developing a remote monitoring system, we have constructed a PP01-*luxI* phage that in conjunction with bioreporter cells is capable of specifically detecting *E. coli* O157:H7. Control assays demonstrated that *E. coli* OHHLux bioreporters do not produce significant light when alone or mixed with LB, phage PP01 or *E. coli* O157:H7. In pure culture, the phage-reporter system is able to detect mid-level (>10^4) concentrations of non-toxigenic *E. coli* O157:H7 in 2 h or less (Fig. 13, Table 7). The luminescence produced by *E. coli* OHHLux bioreporter cells increases rapidly for the first 2 h, then levels off but remains steady for over 20 h (Fig. 13). To detect lower concentrations of *E. coli* O157:H7 (10 – 10^3 CFU/ml), a pre-incubation step is necessary increasing the total assay time to 5 h. We expect that our system will work on most *E. coli* O157:H7 strains, as PP01 is known to infect a broad range of toxigenic and non-toxigenic strains (Morita et al. 2002b, O'Flynn et al. 2004). We confirmed that our phage-bioreporter system does not cross react with selected non-O157:H7 *E. coli* strains and other tested bacterial species. Only a limited number of
strains were examined because the specificity of both components of this system have already been demonstrated (Morita et al. 2002b, O'Flynn et al. 2004, Ripp 2006).

To demonstrate the feasibility of using this detection system in a food sample matrix, *E. coli* O157:H7 was detected in apple juice. Detection of bacteria directly in apple juice was not possible, even when the pH of the apple juice was adjusted from its initial 3.75 to ~7.0. It appeared that the apple juice interfered with the growth and function of the bioreporter cells, as background luminescence was decreased in these experiments. To avoid this problem without going through lengthy bacterial purification steps, a sample of the contaminated apple juice was diluted into LB prior to the detection assay. Dilution of the sample made a preincubation step necessary, but still allowed for the detection of high concentrations (>10<sup>8</sup>) of *E. coli* O157:H7 in under 3 h. The detection limit in apple juice was higher than in LB, with no detectable luminescence produced in response to concentrations of *E. coli* below 10<sup>4</sup> CFU/ml. At 10<sup>4</sup> CFU/ml, detection was possible in less than 8 h (6 h pre-incubation, 1.5 h assay). Detection limits of 10 CFU/ml or lower are desirable in food matrix assays because the infectious dose of *E. coli* O157:H7 is believed to be 10 – 100 cells (LeBlanc 2003). It is possible that a longer pre-incubation period could decrease the detection limit of this assay. We are now focusing on ways to improve the sensitivity and rapidity of this assay.

The bacteriophage based bioluminescent bioreporter described here represents a unique detection system that couples the specificity of the host-bacteriophage interaction with the *lux* quorum sensing and luminescence machinery. This bioreporter system has been shown in initial testing to have the capability to detect *E. coli* O157:H7 from pure culture at mid-level concentrations (10<sup>4</sup> CFU/ml) without sample preincubation, and at
lower concentrations (down to 10 CFU/ml) after sample preincubation in a 96-well plate assay format. While this assay falls short of the desired 10 CFU/ml detection limit in food matrices, its limit of detection is close to that of existing immunoassays and fluorescent-phage based assays (Goodridge et al. 1999, Deisingh & Thompson 2004, Gehring et al. 2004), and the sensitivity of this assay may be increased through additional genetic modifications of the phage. The assay is demonstrated here in a benchtop format, but the significance of this assay is that it has the potential to be developed into a rapid, low-cost, low-energy, real-time online monitoring system for detection of *E. coli* O157:H7 in industrial and environmental settings.
Part IV: Bacteriophage-amplified bioluminescent sensing of *E. coli O157:H7*
This is a version of a journal article submitted to Analytical and Bioanalytical Chemistry entitled “Bacteriophage-amplified bioluminescent sensing of Escherichia coli O157:H7”.

Abstract

Escherichia coli O157:H7 remains a continuous public health threat, appearing in meats, water, fruit juices, milk, cheese, and vegetables where its ingestion at concentrations perhaps as low as 10 to 100 organisms can result in potent toxin exposure and severe damage to the lining of the intestine. Abdominal pain and diarrhea develop, which in the very young or elderly can progress towards hemolytic uremic syndrome and kidney failure. To assist in the detection of E. coli O157:H7, a recombinant bacteriophage reporter was developed that uses quorum sensing (luxI/luxR) signaling and luxCDABE based bioluminescent bioreporter sensing to specifically and autonomously respond to O157:H7 serotype E. coli. The bacteriophage reporter, derived from phage PP01, was tested in artificially contaminated foodstuffs including apple juice, tap water, ground beef, and spinach leaf rinsates. In apple juice, detection of E. coli O157:H7 at original inoculums of 1 CFU/mL occurred within approximately 16 h after a 6 h pre-incubation, detection of 1 CFU/mL in tap water occurred within approximately 6.5 h after a 6 h pre-incubation, and detection in spinach leaf rinsates using a real-time Xenogen IVIS imaging system resulted in detection of 1 CFU/mL within approximately 4 h after a 2 h pre-incubation. Detection in ground beef was not successful, however, due to the natural occurrence of quorum sensing autoinducer (N-3-(oxohexanoyl)-L-homoserine lactone; OHHL) generating false positive bioreporter signals in the ground beef samples.
Introduction

Bacteriophage, or phage for short, are suggested to be the most numerous organisms on Earth, estimated to reside at population levels approaching $1 \times 10^{31}$. With a life cycle that requires a bacterial host for survival and proliferation, these extensive numbers of phage have cultivated diverse host ranges, extending from very narrow (down to the genus or species level) to very broad (infection across strains, species, and genera). The narrowness of phage host ranges has long been realized as a unique mechanism for identifying bacteria. Phage typing schemes, for example, have been applied since the 1940s for specific identification of numerous bacterial pathogens. Newer detection assays that similarly exploit phage/host specificity have since evolved, typically using the phage as a carrier for a reporter signal that indicates when the phage has attached to and/or infected its unique host bacterium. Firefly luciferase ($luc$) (Sarkis et al. 1995), bacterial luciferase ($luxAB$) (Chen & Griffiths 1996), beta-galactosidase ($lacZ$) (Goodridge & Griffiths 2002), ice nucleation ($inaW$) (Wolber & Green 1990), green fluorescent protein ($gfp$) (Funatsu et al. 2002, Oda et al. 2004), quantum dots (Edgar et al. 2006), and nucleic acid stains (Mosier-Boss et al. 2003) have each been used in this regard for the detection of pathogens including Listeria, Mycobacterium, Salmonella, Staphylococcus, and E. coli in a variety of environmental and foodborne matrices. The ease of manufacturing large quantities of phage, their long shelf-life, and associated low cost further merits their practical application for biological sensing. However, integrated phage signaling mechanisms so far described are tied to assay conditions that require the addition of ancillary substrate(s) and specialized, bulky monitoring equipment for signal observation. To move towards a fully reagentless assay amenable to miniaturized lab-on-
a-chip detection schemes (Islam et al. 2007), we engineered a phage reporter system for E. coli O157:H7 that operates autonomously in its sensory capacity. The reporter system centers on the luxRICDABE genetic operon derived from the bioluminescent marine bacterium Vibrio fischeri (Meighen 1994). The luxAB component of this operon encodes for a bacterial luciferase that generates 490 nm bioluminescent light signals when provided with oxygen, FMNH₂, and an aldehyde substrate synthesized by the luxCDE gene complex. The luxI and luxR genes are involved in a cell-to-cell communication network referred to as quorum sensing (Miller & Bassler 2001). The luxI gene synthesizes the autoinducer N-3-(oxohexanoyl)-L-homoserine lactone (OHHL). Freely diffusible OHHL interacts in a cell density dependent manner within the population of V. fischeri cells to activate the regulatory protein LuxR. Activated LuxR subsequently enhances transcription of the lux genes, thereby generating an autoamplified bioluminescent signaling event.

For the specific detection of E. coli O157:H7, a phage, referred to as PP01, was engineered to contain the luxI gene within its genome (thus referred to as PP01-luxI) (Brigati et al. 2007). Upon host infection, the luxI gene is inserted into and expressed by the E. coli cell which then begins synthesizing OHHL. As the OHHL autoinducer molecules diffuse into the extracellular environment, they interact with an OHHL specific bioluminescent bioreporter cell containing the luxR and luxCDABE genes (Ripp et al. 2006). The LuxR protein is activated, transcription of the luxCDABE genes are enhanced, and the bioluminescent bioreporter cell emits bioluminescent light. Thus, the initial phage infection event leads to an autoamplified chemical response that is detected by a luxRCDABE based bioluminescent bioreporter (Fig. 1, Chapter I). To direct this
assay towards foodborne pathogen monitoring applications, we have here demonstrated the utility of PP01-*luxI* reporter phage in the detection of *E. coli* O157:H7 in artificially contaminated apple juice, tap water, and ground beef using a high throughput 96-well microtiter plate format. A real-time bioluminescent imaging assay using a Xenogen IVIS Lumina CCD camera was additionally demonstrated for the detection of *E. coli* O157:H7 in spinach leaf rinsates.

**Materials and methods**

**Bacterial strains and bacteriophage**

Nontoxigenic *E. coli* O157:H7 (ATCC 43888) was used in all experimental procedures and enumerated on Sorbitol MacConkey agar plates when necessary (17 g peptone, 10 g sorbitol, 5 g NaCl, 3 g polypeptone, 1.5 g bile salts, 0.03 g neutral red, 1.0 mg crystal violet, 13.5 g agar per l H₂O, pH 7.0). The reporter phage, PP01-*luxI*, has been previously described (Brigati et al. 2007). Its parent phage, PP01, is highly specific towards strains of *E. coli* O157:H7 and has been shown to not infect other *E. coli* serotypes or other bacterial genera (Morita et al. 2002a, O'Flynn et al. 2004). The bioluminescent bioreporter strain used in this study was *E. coli* OHHLux, which has also been previously described (Ripp et al. 2006). Briefly, OHHLux bioreporters contain the *luxR* and *luxCDABE* genes under the control of the *lux* operon promoter. They bioluminescently respond to OHHL autoinducer within a concentration range from 10 nmol/l to 50 µmol/l.
96-well microtiter plate sampling formats for pure culture, apple juice, tap water, and ground beef detection assays

*E. coli* O157:H7 was tested in a high throughput 96-well microtiter plate format in pure culture and in artificially contaminated apple juice and tap water. For pure culture tests, an overnight culture of *E. coli* O157:H7 was inoculated 1:10 in Luria-Bertani broth (LB; 10 g tryptone, 5 g yeast extract, 10 g NaCl per L H2O, pH 7.0) and grown to an optical density at 600 nm (OD$_{600}$) of 0.26 (~1 × 10$^8$ CFU/ml) at 37 ºC. A 1:10 dilution series, providing cell concentrations ranging from 1 × 10$^7$ to 1 CFU/ml, and a control consisting only of LB, was then prepared in triplicate in 2 mL volumes of LB in a 96-deep well microtiter plate (Abgene 2.2 ml, Thermo Fisher Scientific, Waltham, MA). The use of 96-deep well and standard well microtiter plates throughout these experiments permitted assimilation of the assay with a Beckman Coulter Biomek FX (Fullerton, CA) automated liquid handing workstation for accommodation of high throughput sample processing. Starting concentrations were always verified by dilution plating in triplicate on Sorbitol MacConkey agar plates. The deep well plate was sealed with a Breath-Easy membrane (Diversified Biotech, Boston, MA) and incubated at 40 ºC at 180 rpm for 6 h. The plate was then centrifuged (3750 rpm), the supernatant discarded, the pellets resuspended in 100 µl LB, and then transferred to a 96-well microtiter plate (Microfluor white flat bottom, Thermo Fisher Scientific). To each well of the microtiter plate was then additionally added the PP01-*luxI* reporter phage and the OHHLux bioluminescent bioreporters. The PP01-*luxI* reporter phage were prepared on top agar overlays (Silhavy et al. 1984) and stored refrigerated at stock concentrations of 1 × 10$^{10}$ PFU/ml. Each microtiter plate well received 100 µl of this PP01-*luxI* reporter phage stock (~1 × 10$^9$
PFU/ml final concentration). The OHHLux bioluminescent bioreporter cells were grown in LB at 37 °C to an OD$_{600}$ of 0.35 (~$1 \times 10^8$ CFU/ml) and 50 µL transferred to each microtiter plate well (~$5 \times 10^6$ CFU/ml final concentration). The plate was then sealed with a Breath-Easy membrane and 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 incubation set at 27 °C and shaking set at ‘fast’ speed.

For contaminated apple juice experiments, *E. coli* O157:H7 was grown in LB at 37 °C to an OD$_{600}$ of 0.26, centrifuged at 6000 rpm for 10 min, and washed and resuspended in an equal volume of store purchased pasteurized apple juice. A 1:10 dilution series was then prepared in triplicate 25 mL volumes of apple juice down to 1 CFU/ml and placed at 4 °C overnight. A control of apple juice without an *E. coli* inoculum was similarly placed at 4 °C. For experiments in which there was no pre-incubation, samples were centrifuged the next day, resuspended in 100 µl LB, and combined in a 96-well microtiter plate with 100 µl PP01-*luxI* reporter phage and 50 µl OHHLux bioluminescent bioreporter cells. Plates were monitored for bioluminescence in the Synergy2 as described above. For experiments in which there was a pre-incubation step, samples were centrifuged the next day, resuspended in 2 ml LB in a 96-deep well plate, and incubated with shaking (180 rpm) for 6 h at 40 °C. The deep well plate was then centrifuged and resulting pellets resuspended in 100 µl LB. After transferring to a standard 96-well plate, 100 µl PP01-*luxI* reporter phage and 50 µL OHHLux bioluminescent bioreporters were added and the assay performed as described above.

Unadulterated tap water was artificially contaminated with *E. coli* O157:H7 exactly as described for the apple juice and allowed to sit overnight at 4 °C. Subsequent
dilutions, incubations, plate transfers, and bioluminescent monitoring were unchanged from the apple juice experiments.

Grocery store purchased 93% lean ground beef was weighed in 25 g portions and placed in triplicate sterile 50 ml tubes. A dilution series (1 × 10⁷ to 1 CFU/ml) of E. coli O157:H7 was prepared in LB and 2.5 ml added to each tube. Tubes were then vortexed and allowed to sit overnight at 4 °C. The next day, the contents of each tube were deposited in separate stomacher bags (1650 ml Filtra Bags, Thermo Fisher Scientific) along with an additional 100 ml LB and stomached (Seward Stomacher 400) for 1 min. Resulting liquid was removed, placed in sterile 250 ml flasks, and incubated at 40 °C with shaking (180 rpm) for 6 h. A 10 ml aliquot was then removed from each flask, centrifuged for 10 min at 6000 rpm, and resulting pellets resuspended in 2 ml phosphate buffered saline (PBS; 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, 0.24 g KH₂PO₄ per l H₂O, pH 7.4). To isolate E. coli O157:H7 from the media, 20 µl of anti-E. coli O157:H7 immunomagnetic beads (Dynal Dynabeads, Invitrogen, Carlsbad, CA) were added to these 2 ml volumes, gently mixed for 15 min at room temperature, magnetized for 10 min, washed twice in PBS with subsequent magnetic separation, and finally resuspended in 100 µl of LB. Each 100 µl volume was then transferred to a 96-well microtiter plate along with 100 µl of PP01-luxI reporter phage and 50 µl OHHLux bioluminescent bioreporters, as explained above. The plate was then sealed and monitored for bioluminescence in the BioTek Synergy2 (20 min intervals, 1 sec/well integration time, 27 °C, ‘fast’ shaking).

All experiments included triplicate control wells consisting of pairwise combinations of each component of the assay (i.e., E. coli + PP01-luxI reporter phage, E.
coli + OHHLux bioluminescent bioreporters, PP01-luxI reporter phage + OHHLux, etc.) as well as each component individually. The highest background was consistently generated in control wells containing only the OHHLux bioluminescent bioreporters, thus, rather than graphing multiple control lines, these data were always used as the representative control. These data were also always used for statistical determination of significant differences. A bioluminescent measurement was considered significant if it achieved an intensity two standard deviations (2σ) above these control values.

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

Real-time imaging and quantification of bioluminescence in artificially contaminated spinach was performed with a Xenogen IVIS Lumina CCD imaging system (Alameda, CA). *E. coli* O157:H7 was grown in LB at 37 °C to an OD_{600} of 0.26, centrifuged at 6000 rpm for 10 min, washed, and resuspended in an equal volume of sterile distilled water. A 1:10 dilution series was then prepared to produce a range of *E. coli* concentrations from $1 \times 10^7$ to 1 CFU/ml in 100 mL volumes of sterile distilled water. Store purchased ready-to-eat spinach leaves were weighed in 4 g portions and each portion submerged in a dilution tube and allowed to sit overnight at 4 °C. A control tube contained spinach leaves in sterile distilled water void of an *E. coli* O157:H7 inoculum. The next day, spinach leaves were removed from each dilution tube, placed in sterile petri dishes, and dried for 30 min with covers removed in a sterile fume hood. Each 4 g portion of spinach was then transferred to sterile 100 mL flasks containing 20 ml LB and incubated for 3 h at 40 °C with shaking at 180 rpm. The entire contents of each flask was then individually placed in stomacher bags and stomached for 2 min. Each liquid portion was removed, centrifuged for 10 min at 6000 rpm, and the resulting
pellets resuspended in 1.5 ml of LB. Anti-\textit{E. coli} O157:H7 immunomagnetic beads (20 µL) were added to these 1.5 ml volumes, gently mixed for 10 min at room temperature, and magnetized for 3 min. Captured magnetic bead/\textit{E. coli} complexes were subsequently resuspended in 1.1 ml of LB and 100 µl removed, diluted, and plated on Sorbitol MacConkey agar plates in triplicate to determine actual \textit{E. coli} O157:H7 concentrations. The remaining 1 mL from each tube was transferred to separate 35 mm petri dishes to which was added 1 ml of PP01-\textit{luxI} reporter phage and 0.5 mL OHHLux bioluminescent bioreporters (prepared as described above). Petri plates were placed in the light-tight Lumina imaging chamber set at 27 ºC and monitored for bioluminescence emission every 5 min at an integration time of 1 min.

\section*{Results}

\textbf{Determination of \textit{E. coli} O157:H7 inoculum concentrations}

In all experiments described below, the initial inoculum of \textit{E. coli} O157:H7 was obtained from a culture grown to an OD$_{600}$ of 0.26. This OD yielded an average \textit{E. coli} concentration across all experiments performed of 1.3 (± 0.03) $\times$ 10$^8$ CFU/ml. Consequent 1:10 dilutions prepared from these cultures formed the 1 $\times$ 10$^7$ to 1 CFU/ml dilution series used in all experiments, and plating of these cultures showed similarly tight standard deviations except at 1 CFU/ml where viable plating often failed to yield colonies, as would be expected at this low of a concentration. Thus, rather than noting each specific \textit{E. coli} concentration for each experiment, we have simply stated the concentrations as a range that ultimately approximates to 1 $\times$ 10$^7$ to 1 CFU/ml. Although
it was never known from plate counts if the 1 CFU/ml samples actually contained cells, their bioluminescence emission above controls void of cells clearly established that cells were indeed present.

**Detection of *E. coli* O157:H7 in pure culture**

When inoculated into LB as a pure culture, *E. coli* O157:H7 was detectable after 2 (± 0.3) h at a concentration of $1 \times 10^7$ CFU/ml (Fig. 15). The lowest detectable concentration not requiring a pre-incubation step was $1 \times 10^3$ CFU/ml, where detection occurred within 5 (± 0.3) h of assay initiation. Below this concentration, direct detection of *E. coli* populations was not possible, and therefore a pre-incubation step was added to increase bacterial levels. Our aim was to detect 1 CFU/ml, and the smallest amount of pre-incubation time necessary to do so was determined to be 6 h (data not shown). Since in real-world samples the starting concentration of cells would be unknown, and one would always need to direct the assay towards the detection of 1 CFU/ml, a 6 h pre-incubation became a standard component of the assay. These additional 6 h of pre-incubation then permitted detection of 100 CFU/ml within 1 (± 0.3) h (~7 h total), 10 CFU/ml within 2.5 (± 0.3) h (~8.5 h total), and 1 CFU/ml within 4.5 (± 0.7) h (~10.5 h total). To account for preparation of cultures and loading of the 96-well microtiter plate, an additional 0.5 h can be added to all detection times to establish the total assay time. Again, once this 0.5 h preparation time is complete and the microtiter plate has been placed in the light reader, no other user interaction is required, except for data analysis at the termination of the assay run. Thus, assays can be completed within a normal 8 h work day with results available within 24 h.
E. coli at concentrations ranging from $1 \times 10^7$ to $1 \times 10^3$ CFU/ml could be detected directly, without pre-incubation, within approximately 2 to 5 h, respectively, of assay initiation. Lesser concentrations (100 to 1 CFU/ml) required a minimum pre-incubation of 6 h at 40 °C (black bars) to elevate cell numbers to detectable levels. This resulted in total detection times ranging from 7 h (100 CFU/ml) to 10.5 h (1 CFU/ml).

**Figure 15. Establishment of the bioluminescent response in a pure culture 1:10 dilution series of E. coli O157::H7**

E. coli at concentrations ranging from $1 \times 10^7$ to $1 \times 10^3$ CFU/ml could be detected directly, without pre-incubation, within approximately 2 to 5 h, respectively, of assay initiation. Lesser concentrations (100 to 1 CFU/ml) required a minimum pre-incubation of 6 h at 40 °C (black bars) to elevate cell numbers to detectable levels. This resulted in total detection times ranging from 7 h (100 CFU/ml) to 10.5 h (1 CFU/ml).
Detection of *E. coli* O157:H7 in apple juice

Twenty-five ml volumes of store purchased pasteurized apple juice were artificially inoculated with *E. coli* O157:H7 from $1 \times 10^7$ to 1 CFU/ml and allowed to set at 4 ºC overnight. These samples were then centrifuged, resuspended in LB, and either used directly in the assay or pre-incubated for 6 h at 40 ºC prior to assay incorporation. Without pre-incubation, detection from $1 \times 10^5$ to $1 \times 10^7$ CFU/ml was possible within less than 5 h (Fig. 16). However, concentrations below $1 \times 10^5$ CFU/ml failed to initiate bioluminescent signals unless a pre-incubation step was included to increase threshold *E. coli* levels. With pre-incubation, it was then possible to detect down to 100 CFU/ml in 5 (± 0.7) h. Detection of 10 or 1 CFU/ml, however, remained unsuccessful. Extending the pre-incubation time by several hours did permit detection at these lowest concentrations, but additionally extended the assay time beyond the 24 h limits that we wished to maintain (data not shown). Therefore, sample size was doubled from 25 ml to 50 ml apple juice while preserving all other assay parameters. This then permitted detection of original 10 and 1 CFU/ml inoculums within less than 24 h (19 (± 0.7) h at 10 CFU/ml and 22 (± 0.7) h at 1 CFU/ml).

Detection of *E. coli* O157:H7 in tap water

*E. coli* O157:H7 inoculums ranging from $1 \times 10^7$ to 1 CFU/ml were added to unadulterated tap water in 25 ml volumes and processed as for the apple juice. The $1 \times 10^7$ to $1 \times 10^5$ CFU/ml cultures were detectable without pre-incubation within 5 (± 0.3) to 7 (± 0.7) h, respectively, of assay initiation (Fig. 17). Lower concentrations again
At elevated concentrations (1 \times 10^5 to 1 \times 10^7 CFU/ml), \textit{E. coli} O157:H7 was detectable in 25 ml sample volumes without pre-incubation within less than 5 h. At all lower concentrations, a 6 h pre-incubation step (black bars) was necessary to bring \textit{E. coli} to threshold detectable levels. *Detection at 10 and 1 CFU/ml required that sample volumes be increased from 25 ml to 50 ml, thereby achieving detection in approximately 19 and 22 h, respectively.

Figure 16. Bioluminescent detection of \textit{E. coli} O157:H7 in artificially contaminated apple juice
Concentrations below $1 \times 10^5$ CFU/ml required a 6 h pre-incubation (black bars) at 40 °C to elevate cells to detectable levels. This then enabled detection down to CFU/ml in a total assay time of 12.3 (± 0.7) h.

Figure 17. Response time profile of bioluminescence emission in 25 ml sample volumes of *E. coli* O157:H7 contaminated tap water
required a 6 h pre-incubation, providing total detection times ranging from 8.3 (± 0.7) h at $1 \times 10^4$ CFU/ml up to 12.3 (± 0.7) h at 1 CFU/ml.

**Detection of *E. coli* O157:H7 in ground beef**

Only the highest concentrations ($1 \times 10^7$ and $1 \times 10^6$ CFU/ml) of *E. coli* O157:H7 were detectable in artificially contaminated ground beef samples, with detection occurring within 4 – 6 h after a 6 h pre-incubation (data not shown). At all other concentrations, no significant differences existed between sample and control bioluminescence emissions. Control wells containing OHHLux bioluminescent bioreporter cells often generated background bioluminescence 1000X that seen in LB, apple juice, and tap water experiments. The presence of intrinsic OHHL in ground beef has been reported (Bruhn et al. 2004), and it is likely that this accounts for the extensive background induction of the OHHLux bioluminescent bioreporter and subsequent inability to distinguish between it and actual induction by *E. coli* O157:H7 presence. Thus, the assay as it currently operates cannot adequately detect *E. coli* O157:H7 in ground beef, but could be modified to do so (see Discussion).

**CCD imaging of *E. coli* O157:H7 in spinach leaf rinsates**

A Xenogen IVIS Lumina CCD imaging camera was used to monitor *E. coli* O157:H7 induced bioluminescence in real time in spinach leaf rinsates. Although these experiments began with a $1 \times 10^7$ to 1 CFU/ml dilution series, the absorption of *E. coli* onto spinach leaves and the subsequent processing and incubation steps was not expected to maintain a 1:10 stepwise concentration of cells throughout the experimental procedure. Therefore, immediately after immunomagnetic separation of *E. coli* O157:H7 from the
rinsate, each sample was plated to determine actual cell numbers directly prior to placing
the samples in the IVIS imaging chamber. This resulted in final cell concentrations, in
sequential order corresponding to the original $1 \times 10^7$ to 1 CFU/ml dilution series, of $5.6 \times 10^8, 2.7 \times 10^9, 6.9 \times 10^7, 4.2 \times 10^6, 8.8 \times 10^5, 1.5 \times 10^4, 1.3 \times 10^3$, and $2.3 \times 10^4$ CFU/ml. At the highest final \textit{E. coli} concentration of $2.7 \times 10^9$ CFU/ml (derived from the original $1 \times 10^6$ CFU/ml inoculum), significant bioluminescence was observed within 1.4 h after the 2 h pre-incubation (Fig. 18). The lowest final \textit{E. coli} concentration ($1.3 \times 10^3$ CFU/ml, derived from the original 1 CFU/ml inoculum) generated measurable bioluminescence 3.6 h after the 2 h pre-incubation.

**Discussion**

The application of bacteriophage for selective identification of bacterial pathogens presents a powerful diagnostic for microbial detection and control. In addition to being highly specific for their target hosts, phage also infect only metabolically active cells, are self-replicating, are as ecologically diverse as their bacterial counterparts, and can be prepared at very high titer for very low cost with extensive shelf-life. In these studies, the testing of the diagnostic reporter phage PP01-\textit{luxI} was performed in pure culture, apple juice, tap water, ground beef, and spinach leaf rinsate, with the goal of detecting \textit{E. coli} O157:H7 at 1 CFU/ml in less than 24 h. Although in these particular studies only the nontoxigenic \textit{E. coli} ATCC 43888 strain was used, we have previously demonstrated that PP01-\textit{luxI} likewise infects and bioluminescently identifies other toxic O157:H7 serotypes, for example ATCC strains 43889, 43894, 43895, and 700927 (Brigati et al. 2007).
The upper panel depicts time series images of 35 mm petri dishes containing rinsates of varying *E. coli* O157:H7 concentrations (A (○), $5.6 \times 10^8$; B (●), $2.7 \times 10^9$; C (□), $6.9 \times 10^7$; D (■), $4.2 \times 10^6$; E (△), $8.8 \times 10^5$; F (solid line), $1.5 \times 10^4$; G (▲), $1.3 \times 10^3$; H (gray dotted line), $2.3 \times 10^4$ CFU/ml; I (+), control) combined with PP01-*luxI* reporter phage and OHHLux bioluminescent bioreporters, and the graph in the lower panel displays the average bioluminescence emission over time for each plate. The earliest bioluminescent signal was observed 1.4 h after the 2 h pre-incubation at a final cell density of $2.7 \times 10^9$ CFU/ml (plate B). Cell densities corresponding to original inoculums of 100, 10, and 1 CFU/ml (plates F, G, and H, respectively) generated observable bioluminescence within 3 – 4 h following the 2 h pre-incubation.

Figure 18. Real-time imaging of bioluminescence emission from rinsates from *E. coli* O157:H7 contaminated spinach leaves
It does not infect other *E. coli* serotypes, other *E. coli* strains, or other bacterial genera (Morita et al. 2002a, O'Flynn et al. 2004, Brigati et al. 2007).

Detection of *E. coli* O157:H7 was successful in all sample matrices except for ground beef. Pure culture LB broth samples established optimal detection of 1 CFU/ml in approximately 10.5 h, inclusive of a necessary 6 h pre-incubation enrichment to elevate *E. coli* O157:H7 concentrations (Fig. 15). Apple juice and tap water similarly required 6 h pre-incubations, resulting in total detection times of approximately 22 h and 12.5 h, respectively, at initial *E. coli* inoculums of 1 CFU/ml in sample volumes of 50 mL apple juice or 25 ml tap water (Figs. 16 and 17). The additional volume and time necessary for detection in apple juice is likely due to the documented effects of its acidity (pH ~ 4) on slowing *E. coli* growth (Koodie & Dhople 2001). Detection of elevated *E. coli* concentrations (≥ 1 × 10⁵ CFU/ml) in apple juice and tap water could be achieved directly without pre-incubation in less than 5 and 7.5 h, respectively.

Detection of *E. coli* O157:H7 in ground beef was successful at high *E. coli* inoculums (≥ 1 × 10⁶ CFU/ml), but significant bioluminescence emission, as compared to control ground beef samples void of an artificial *E. coli* inoculum, could not be established at any lower *E. coli* concentrations. It is known that ground beef inherently contains OHHL autoinducer, thought to be primarily synthesized by resident *Enterobacteriaceae*, principally *Hafnia alvei* (Bruhn et al. 2004). Since the PP01-*luxI* assay relies on a bioluminescent bioreporter organism sensitive to OHHL, samples harboring OHHL from secondary sources will erroneously activate the bioreporter resulting in false positive signaling. We have demonstrated this vulnerability in the presence of other OHHL producing bacteria such as *Yersinia enterocolitica* and *Erwinia*
carotovora, as well (Ripp et al. 2006, Brigati et al. 2007). The need for controls containing the OHHLux bioreporter alone combined with test sample are therefore mandatory to identify these false positives. Alternatively, an effective phage reporter for E. coli in ground beef can be developed using other quorum sensing regulatory circuits (Miller & Bassler 2001). For example, the rhlI/rhlR quorum sensing network from Pseudomonas aeruginosa could be substituted for luxI/luxR to create a phage reporter/bacterial bioreporter system sensitive to butanoyl-L-homoserine lactone (BHL) autoinducer rather than OHHL autoinducer. This additionally should allow for multiplexed bacterial target detection via insertion of different autoinducer signaling cascades within each phage reporter.

Detection of E. coli O157:H7 in spinach leaf rinsates was performed with a Xenogen IVIS Lumina CCD camera using real-time bioluminescence imaging (BLI) technology. The preponderance of BLI techniques center around in vivo non-invasive imaging of bioluminescent and/or fluorescent signals in animal models, but its extreme sensitivity begs for application in other signal detection practices, of which pathogen monitoring in foodstuffs has seen only modest pursuit (Maoz et al. 2002). In these studies, as few as 1 CFU/ml E. coli could be detected within approximately 6 h, inclusive of a 2 h pre-incubation (Fig. 18).

Although diagnostic phage have previously been exploited for foodborne pathogen detection, their typical reliance on reporter gene systems such as green fluorescent protein or reagent constrained luxAB can be detrimental due to excitation light source requirements or ancillary substrate additions. The use of the complete luxCDABE cassette described here, in conjunction with autoamplified quorum sensing signaling,
provides a unique reagentless diagnostic that we have shown requires minimal user set-up (~ 0.5 h) and downstream interaction, and suitably interfaces with robotic liquid handling workstations such as the Biomek FX when high throughput analysis is required. The additional implementation of these phage reporter systems with real-time BLI further enhances the utility of these assays.
Chapter V: Conclusions
Recent outbreaks of disease due to *E. coli* O157:H7 have pushed the need for monitoring of food and water supplies to the forefront. Given the obvious public health importance of this pathogen, a detection method that is rapid, specific, and capable of detection at low concentrations is needed to prevent outbreaks or at least quickly detect and limit the number of individuals affected. There are a variety of methods available for the detection of *E. coli* O157:H7 but most are slow, costly and/or complex because they require a significant amount of sample manipulation. The bacteriophage based bioreporter system is an alternative to these detection methods.

The development of two phage based *luxI* bioreporters (Ch. II) as a general *E. coli* detection method and (Ch. III and IV) as a specific detection method for the important bacterial pathogen *E. coli* O157:H7 were examined in this work (Ripp 2006, Brigati et al. 2007, Ripp Submitted 2007). The *luxI* gene from *V. fischeri* was placed into the phage genomes which upon infection of the host led to production of quorum sensing molecules, specifically OHHL. OHHL diffuses extracellularly and activates the *E. coli* OHHLux bioreporter cells containing the *luxR* and *luxCDABE* genes. OHHL binds *luxR* leading to transcription of *luxCDABE* to produce a detectable signal in the form of bioluminescence and yielding a positive assay result.

The system requires a simple three step protocol consisting of the addition of reporter phage, bioreporter, and target cells (or sample). No other reagents or manipulations are required other than incubation and light measurement, making the assay amenable to automated high throughput monitoring. The preliminary testing reported here demonstrates the ability of *luxI*-incorporated bacteriophage to detect target
microbes in a simple and cost-effective microtiter plate format. The general *E. coli* system with the λ<sub>lex</sub> phage was applied to pure culture and lettuce rinsates resulting in detection times ranging from less than 3 hours for high cell concentrations (10<sup>8</sup> CFU/ml) to well under 24 hours for lower cell densities (1-130 CFU/ml). The *E. coli* O157:H7 specific system with PP01-*luxI* phage was tested in pure culture, apple juice, tap water, ground beef, and spinach leaf rinsates. Detection of *E. coli* O157:H7 was successful in all sample matrices except for ground beef. Assay detection times for original inoculums 1 CFU/ml ranged from 10.5 h in pure culture, 12.5 h for tap water and 22 h for apple juice. More recent BLI technology has demonstrated real-time detection of *E. coli* O157:H7 at concentrations of 1 CFU/ml in spinach leaf rinsates in under 6 h.

While the bacteriophage based bioreporter system does present a viable option for pathogen detection, it has some issues to overcome. Although the phage reporters have shown unique specificity for their host, synthesis of OHHL by other bacteria can induce bioluminescence by the OHHLux bioreporter. The presence of these OHHL producing microorganisms in a sample would therefore generate false positive signals. Thus it is imperative that assays include a control consisting of bioreporter alone added to the sample to identify if there is intrinsic OHHL production. The emergence of phage resistant target hosts may also occur reducing the system’s effectiveness, but the use of multi-phage cocktails and the natural reversion back to phage sensitivity often occurs (O'Flynn *et al.* 2004). Additionally, OHHL lactonases, AHL inactivating enzymes, could interfere with detection if present in high enough concentrations. Also the assay would likely not detect VBNC cells due to the fact that phage infection is very sensitive to the
physiologic status of the host cell, thus the reporter phage would be unable to infect and use the host cell machinery to produce the OHHL required for this system.

The application of bacteriophage for the precise identification of bacterial pathogens is a powerful diagnostic for microbial detection. This technology is versatile and can be applied to a wide variety of sample matrices. The assay could be improved by increasing sensitivity and total detection time through additional genetic modifications to the reporter phage. Furthermore, phage reporters can be developed using other quorum sensing systems other than luxI/luxR from *V. fischeri* (Miller & Bassler 2001). Creating a phage reporter/bacterial bioreporter system that is responsive to autoinducers other than OHHL could allow for multiplexed bacterial target detection through insertion of different autoinducer signaling cascades within each phage reporter.
References


Growth Indicator Tube (MGIT) system. Diagnostic microbiology and infectious disease 45:53-61


CDC (1996b) Outbreak of Escherichia coli O157:H7 infections associated with drinking unpasteurized commercial apple juice -- British Columbia, California, Colorado, and Washington, October 1996. MMWR 45:975


Gambello MJ, Kaye S, Iglewski BH (1993) LasR of *Pseudomonas aeruginosa* is a transcriptional activator of the alkaline protease gene (apr) and an enhancer of exotoxin A expression. Infection and immunity 61:1180-1184


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Hastings JW (1978) Bacterial bioluminescence light emission in the mixed function oxidation of reduced flavin and fatty aldehyde. CRC critical reviews in biochemistry 5:163-184


Hay AG, Rice JF, Applegate BM, Bright NG, Sayler GS (2000) A bioluminescent whole-cell reporter for detection of 2, 4-dichlorophenoxyacetic acid and 2,4-dichlorophenol in soil. Applied and environmental microbiology 66:4589-4594


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enterocolitica mediating the synthesis of two N-acylhomoserine lactone signal molecules. Molecular microbiology 17:345-356


Vita

Courtney was born on January 6, 1982 in Ypsilanti, MI. She received her B.S. in Biological Sciences with a concentration in Microbiology from the University of Tennessee, Knoxville. Then she pursued her Master of Science degree in Microbiology at the Center for Environmental Biotechnology at the University of Tennessee under Dr. Gary Sayler and Dr. Steven Ripp.