AFRL-ML-TY-TP-2002-4523

DETECTION OF PATHOGENIC ORGANISMS IN FOOD, WATER AND BODY (POSTPRINT)

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27 June 2002

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE *(DD-MM-YYYY)*: 27 June 2002

2. REPORT TYPE: Journal Article - POSTPRINT

3. DATES COVERED *(From - To)*:

4. TITLE AND SUBTITLE: Detection of Pathogenic Organisms in Food, Water and Body (POSTPRINT)

5a. CONTRACT NUMBER:

5b. GRANT NUMBER:

5c. PROGRAM ELEMENT NUMBER:

5d. PROJECT NUMBER:

5e. TASK NUMBER:

5f. WORK UNIT NUMBER: M11R2000

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8. PERFORMING ORGANIZATION REPORT NUMBER:

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
%Air Force Research Laboratory
Materials and Manufacturing Directorate
Airbase Technologies Division
139 Barnes Drive, Suite 2
Tyndall Air Force Base, FL 32403-5323

10. SPONSOR/MONITOR'S ACRONYM(S): AFRL/MLQ

11. SPONSOR/MONITOR'S REPORT NUMBER(S): AFRL-ML-TY-TP-2002-4523

12. DISTRIBUTION/AVAILABILITY STATEMENT
DISTRIBUTION A. Approved for public release; distribution unlimited.
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13. SUPPLEMENTARY NOTES
Distribution Code 20: JOURNAL ARTICLES; DTIC USERS ONLY. Document contains color images. Published in Proc. SPIE 4722, Chemical and Biological Sensing III, 26 (June 27, 2002); doi:10.1117/12.472259.

14. ABSTRACT
The construction of specific bioluminescent bacteriophage for detection of pathogenic organism can be developed to overcome interferences in complex matrices such as food, water and body fluids. Detection and identification of bacteria often require several days and frequently weeks by standard methods of isolation, growth and biochemical test. Immunoassay detection often requires the expression of the bacterial toxin, which can lead to non-detection of cells that may express the toxin under conditions different from testing protocols. Immunoassays require production of a specific antibody to the agent for detection and interference by contaminants frequently affects results. PCR based detection may be inhibited by substances in complex matrices. Modified methods of the PCR technique, such as magnetic capture-hybridization PCR (MCH-PCR), appear to improve the technique by removing the DNA products away from the inhibitors. However, the techniques required for PCR-based detection are slow and the procedures require skilled personnel working with labile reagents. Our approach is based on transferring bioluminescence (lux) genes into a selected bacteriophage. Bacteriophages are bacterial viruses that are widespread in nature and often are genus and species specific. This specificity eliminates or reduces false positives in a bacteriophage assay. The phage recognizes a specific receptor molecule on the surface of a susceptible bacterium, attaches and then injects the viral nucleic acid into the cell. The injected viral genome is expressed and then replicated, generating numerous exact copies of the viral genetic material including the lux genes, often resulting in an increase in bioluminescence by several hundred fold.

15. SUBJECT TERMS
bacteriophage, bioluminescence, and lux genes

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Michael V. Henley
U	U	U	UU	7	19b. TELEPHONE NUMBER *(Include area code)*: 850 283 6050

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Prescribed by ANSI Std. Z39.18

Detection of pathogenic organisms in food, water and body fluids

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ABSTRACT

The construction of specific bioluminescent bacteriophage for detection of pathogenic organism can be developed to overcome interferences in complex matrices such as food, water and body fluids. Detection and identification of bacteria often require several days and frequently weeks by standard methods of isolation, growth and biochemical test. Immunoassay detection often requires the expression of the bacterial toxin, which can lead to non-detection of cells that may express the toxin under conditions different from testing protocols. Immunoassays require production of a specific antibody to the agent for detection and interference by contaminants frequently affects results. PCR based detection may be inhibited by substances in complex matrices. Modified methods of the PCR technique, such as magnetic capture-hybridization PCR (MCH-PCR), appear to improve the technique by removing the DNA products away from the inhibitors. However, the techniques required for PCR-based detection are slow and the procedures require skilled personnel working with labile reagents.

Our approach is based on transferring bioluminescence (*lux*) genes into a selected bacteriophage. Bacteriophages are bacterial viruses that are widespread in nature and often are genus and species specific. This specificity eliminates or reduces false positives in a bacteriophage assay. The phage recognizes a specific receptor molecule on the surface of a susceptible bacterium, attaches and then injects the viral nucleic acid into the cell. The injected viral genome is expressed and then replicated, generating numerous exact copies of the viral genetic material including the *lux* genes, often resulting in an increase in bioluminescence by several hundred fold.

Key words: bacteriophage, bioluminescence, *and lux* genes

INTRODUCTION

Bioluminescent bioreporter technology history and application to detection of chemicals

Bioreporter technology consists of microbes that have been genetically engineered to detect and quantify specific chemical agents in air, soil, or water by giving off a measurable bioluminescent signal that is proportional to the concentration of the agent. In the process of bacterial degradation of pollutants, the induction and expression level of specific catabolic genes are often controlled by the presence and relative amounts of the pollutant to be catabolized (18). Thus, measurement of the amount of gene expression can be used to infer the relative quantities of bioavailable contaminant in the sensing environment. Direct measurement of catabolic gene expression is often difficult, as no obvious phenotypic change may occur in the organism. Gene expression, however, may be measured by modifying an organism to use contaminant-sensitive promoters to activate genetic machinery to produce a more detectable change in the organism in response to the contaminant, i.e. by producing reporting gene products (10). Presence of contaminant thus promotes the production of gene products that cause a more measurable phenotypic change in the organism, i.e. bioluminescence, fluorescence, etc. In our approach, the bioluminescent reporter genes, *lux,* produce blue-green light (490 nm) which can be measured rapidly and with high sensitivity. The *lux* genes were originally cloned from the marine bacterium *Vibrio fischeri.* The bioluminescent (*lux*) genes from bacteria have been genetically and biochemically characterized. The

Chemical and Biological Sensing III, Patrick J. Gardner, Editor,
Proceedings of SPIE Vol. 4722 (2002) © 2002 SPIE · 0277-786X/02/$15.00

genes, *lux C, D* and *E* encode for a reductase, a transferase and a synthetase, respectively, that constitute the three enzymes from a multi-enzyme fatty acid reductase complex. The *lux A*, gene encodes for the alpha and beta subunits of the enzyme luciferase (6). Luciferase is a generic name for an enzyme that catalyzes a light generating reaction. Luciferases are wide spread found, in bacteria, algae, fungi jellyfish, insects, shrimp, and squid the light produced by these organisms is defined as bioluminescence.

By introducing the genes into the catabolic gene loci of bacteria without native *lux* sequences, the production of 490 nm light from the transformed bacteria is dependent only upon contaminant exposure. The amount of light produced is dependent upon the level of expression of the reporter gene, and thus upon the exposure level to the inducing pollutant.

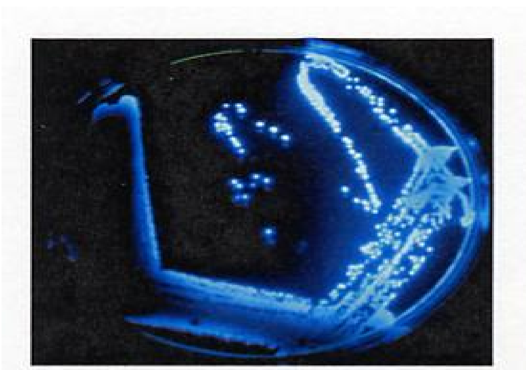

Figure 1. Bioluminescent emitted from individual colonies of reporter bacteria on the surface on an agar plate. The cells were induced with naphthalene vapors for the expression of *lux* genes.

Engineered bacteria containing the *lux* gene cassette have already been implemented as bioreporters for a wide variety of environmental contaminants. As early as 1990, whole cell bioluminescent bioreporters were developed with the appropriate regulated metabolic operons and were shown to function in complex environmental matrices as remote sensors (10). Bioreporters for detection of zinc, cadmium, cobalt, lead, mercury, copper and nickel have also been reported in the literature (5,20). The University of Tennessee pioneered the use of bioluminescence in environmental chemical sensing through development of bioreporter bacterial strains harboring bioluminescent (bacterial) *lux* gene fusions with promoters specific for detection of aromatic hydrocarbons (17,19). Several bioluminescent bioreporters have been constructed at UT-CEB for the detection of naphthalene and salicylate (8,10) BTEX compounds (benzene, toluene, ethyl benzene, xylene) (1) polychlorinated biphenyls (13), dinitrotoluene, 2,4-dichlorophenoxyacetic acid and dichlorophenol (7) and heavy metals (23). Bioreporters have been used to screen for single or several chemically related compounds simultaneously. For example, a bioluminescent bioreporter was constructed at University of Tennessee, Center for Environmental Biotechnolgy for the specific detection of toluene using the promoter from the *tod* operon (1). The bioreporter demonstrated increased bioluminescence with increased concentrations of toluene (1). In addition to toluene, this bioreporter responded to other BTEX compounds and trichloroethylene (2,21) with highly sensitive detection limits of approximately 30 μg/L.

Proof-of-concept for field application of bioreporter technology has been observed in studies demonstrating reproducible bioreporter based bioluminescence in aqueous and soil slurry samples which contained naphthalene, in complex soil leachates, and water-soluble components of jet fuel (8,9). *Pseudomonas* sp. bioreporter bacteria were shown to function as reagent-less, living whole cell biosensors and ultimately the organisms were approved for field-testing by the EPA and were environmentally released at the Y-12 facilities at ORNL (16,19). Bioreporter cells in this controlled field test have maintained functional status in the environment for 3 years. Non-chip-based bioluminescent techniques for spatial characterization of naphthalene and toluene equivalents in contaminated soil at the Natural Attenuation Test Site at Columbus AFB, MS, (24) have been applied with good correlation against GC/MS sample analysis.

Detection of bacterial cells by bioluminescent bioreporters

The *lux* bioreporters described above have been designed to detect and monitor chemical substances. Application of *lux* reporters for pathogenic bacteria associated with food or water, infectious diseases and biological warfare agents requires integrating the *lux* genes into the bacteriophage specific for the bacterial pathogen. Several laboratories have successfully designed bacteriophage *lux* reporters for food borne pathogens (Table 1). Standard microbial methods for identification of unknown bacteria often relies on determination of which bacterial strain is a host for a specific bacteriophage, phage typing. Diagnostic identification of *Mycobacterium tuberculosis, Salmonella, Campylobacter, Staphylococcus aureus, Listeria* and *E. coli* are performed in this manner. (4,14,15).

Bacteriophages are bacterial viruses, which are widespread in nature and often are genus and species specific. Bacteriophages are obligate intracellular parasites consisting of a simple protein coat surrounding the genetic material. The phage recognizes a specific receptor molecule on the surface of a susceptible bacterium attaches and injects the viral nucleic acid into the cell. The injected viral genome is expressed and then replicated to generate numerous exact copies of the viral genome material. Eventually new phage particles fill the cell and are released, as the bacterial cell is lysed (3,12).

Bacterial Pathogen	Detection Limit	Test Source	Reference
Enterobacteriaceae	10 cells/g/cm^2	Surface and meat carcass swabs	11
Escherichia coli species	100 cells/ml	Milk	26
Escherichia coli O157:H7	Not determined	Pure culture	27
Listeria monocytogenes	10 cells/g	Cheese, pudding, cabbage	14
Salmonella species	10 cfu/ml	Eggs	4
Salmonella typhimurium	100 cells/ml	Pure culture	25
Staphylococcus aureus	1000 cfu/ml	Pure culture	15

Table 1. *lux AB*-based bioreporters, which target specific bacterial pathogens.

Bacteriophages exist for all species of bacteria and many have been isolated, identified and characterized for bacterial pathogens. Additional phage can be identified and isolated for any particular bacterial species by standard microbiological methods (3,12)

The *lux* based bioreporters can be constructed in one of two ways, either using the *lux AB* genes or the *lux CDABE*. The *lux AB* genes will only produce light when exogenous decanal is provided to the bioreporter and light will fade once this substrate is depleted. Therefore, *lux AB* bioreporters do not provide independent and continuous sensors, but a single point sensor. The *lux CDABE* genes bioreporter are capable of long term, continuous, quantitative detection of target molecules (6). The UT, CEB is currently developing *lux CDABE* whole cell bioreporters to provide independent and continuous sensors, integrated with bacteriophage specific for pathogenic bacteria. The bioreporter design will incorporate a *lux* gene from *Vibrio fischeri* into the genome of the specific bacteriophage isolated and identified to target a particular pathogenic bacteria. Once the bacteriophage infects the target cell, the *lux* gene is expressed and cell-to-cell communication factors are released. An important feature of the bacteriophage based sensor is the amplification of bacteriophage during the infection process, one phage can infect one target cell, and the phage genome takes over the cell's normal processes and generates hundreds of phage particles intracellular. The phage particles are released from the cell ready to infect other target cell with a repeated round of amplification. Therefore a few target cells can result in thousands of copies of

bacteriophage genome of which each genome copy will generate many communication signals. This amplification allows the sensor to produce a detectable signal from just a few target cells. The communication signal molecule will induce a second neighboring cell containing the complete *lux CDABE* gene cassette to produce a bioluminescent signal (Figure 2).

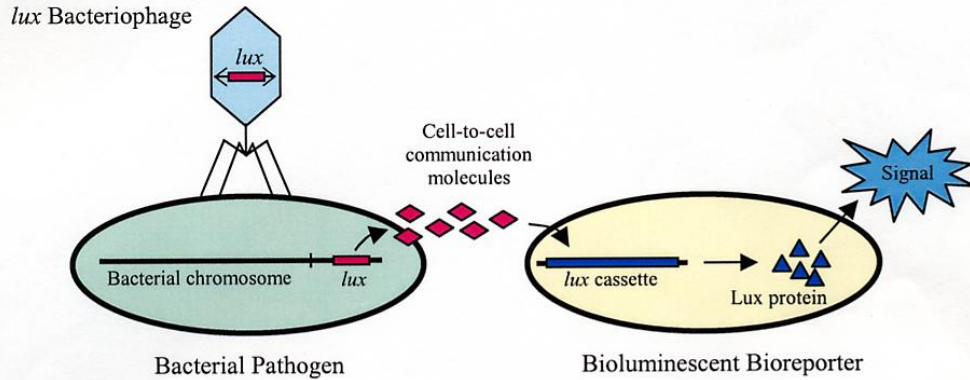

Figure 2. Diagram of a bacteriophage activated bioluminescent bioreporter. The bacteriophages are genetically engineered to contain a *lux* gene involved in a cell-to-cell communication network referred to as quorum sensing. The *lux*-based bacteriophages are highly specific, and infect only one particular type of bacterial pathogen. Infection results in the production of cell-to-cell communication molecules that are taken up by a neighboring bioluminescent bioreporter cell whose *lux* genes are then activated to produce a bioluminescent signal.

Detection of the bioluminescent signal

Many years of effort in multiple scientific fields have focused on methods for detection and measurement of light; therefore we can take advantage of the available methods in our detection system. Optical transducers such as photomultiplier tubes, photodiodes, microchannel plates, or charge-coupled devices are readily available and can be easily integrated into high throughput readers. The need for sensors that are small, rugged and portable to field applications has resulted in the development of battery-operated, hand held photo multiplier units. Scientists at the UT,CEB collaborating with scientists at Oak Ridge National Laboratory have miniaturized optical transducers and successfully developed integrated circuits capable of detecting bioluminescence directly from bioreporter organisms (Figure 3). They accomplished quantitation of the bioluminescent signal by interfacing the bioreporters directly with an Optical Application Specific Integrated Circuit [OASIC] (22,23). The integration of a highly sensitive optical sensor with advanced signal processing techniques reduces the cost and powering requirements of the biosensor by enabling production using standard bulk complementary metal oxide semiconductor (CMOS) chip fabrication processes. The Bioluminescent Bioreporter Integrated Circuit (termed BBIC; Figure 4) concept was patented and is under CRADA development (with DOE and Dynamac support) for ORNL-UT research.

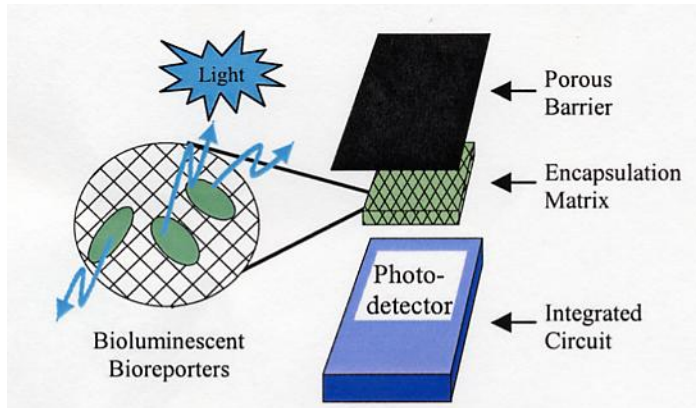

Figure 3. Conceptual BBIC system showing the immobilized bioreporter inserted between a porous layer and the integrated circuit with a photodetector.

University of Tennessee, Center for Enivironmental Biotechnolgy has developed and tested an operating laboratory prototype BBIC that couples the biologically active whole-cell bioreporter matrix with microelectronics for data storage and transmission to form a cost-effective, miniaturized BBIC (2,7,22,23) which permits remote data collection. The whole-cell bioreporter matrix is housed in a metabolically supportive matrix that rejects ambient light while providing analyte/bioreporter interaction. The matrix also prevents release of the microbes into the environment. The BBIC will also have contaminant mapping capabilities through incorporation of a global positioning system (GPS). In this format, BBICs could be hand placed or air-dropped to conduct near real-time surveys and rapidly map contamination over large areas.

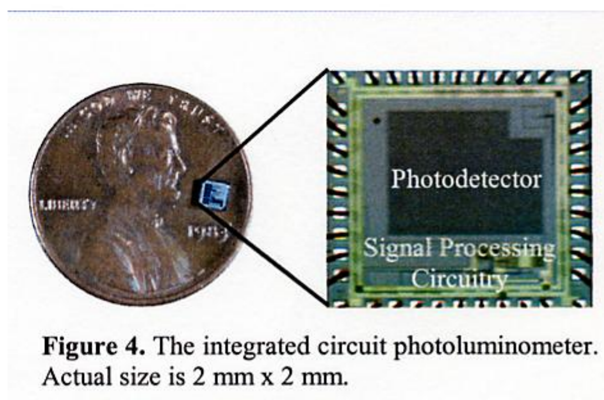

Figure 4. The integrated circuit photoluminometer. Actual size is 2 mm x 2 mm.

Applications

The bioreporter technology will provide a cost effective, robust, quantitative measurement of biological contamination in complex media such as food, water, body fluids and possible sensors for biological contamination on the battlefield. The advanced photonic detection technologies such as the BBIC provides fully automated package for continuous monitors and remote sensors of biological hazards. The whole-cell bioreporter matrix could be immobilized on indicator test strips. The test strips would be applicable as a home water quality indicator, the test kit could be developed to function in much the same way as a home

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