CONF-960848--10

Development And Application Of The *lux* Gene For Environmental Bioremediation

R.S. Burlage*[1], Z. Yang[1], R.J. Palmer[2], and Y. Khang[3]

1. Environmental Sciences Division
Oak Ridge National Laboratory
Oak Ridge, TN 37831-6036

2. Center for Environmental Biotechnology
University of Tennessee
10515 Research drive
Knoxville, TN 37932

3. Yeungnam University
Department of Applied Microbiology
College of Natural Resources
Yeungnam University
Kyongsan, 712-749
Republic of Korea

* Corresponding author

ABSTRACT

Bioremediation is the use of living systems, usually microorganisms, to treat a quantity of soil or water for the presence of hazardous wastes. Bioremediation has many advantages over other remediation approaches, including cost savings, versatility, and the ability to treat the wastes in situ. In order to study the processes of microbial bioremediation, we have constructed bacterial strains that incorporate genetically engineered bioreporter genes. These bioreporter genes allow the bacteria to be detected during in situ processes, as manifested by their ability to bioluminesce or to fluoresce. This bioreporter microorganisms are described, along with the technology for detecting them and the projects which are benefiting from their application.

Keywords: bioluminescence, fluorescence, biotechnology, environment, bioremediation, microbiology

1. Description of the Technology

The technology that will be described here is the use of living cells to indicate when specific biological properties are functioning, i.e., these cells are **bioreporters** of a certain activity. There are two types of bioreporters that will be described here: bioluminescent and fluorescent bioreporters. Bioluminescence is the production of visible light by a biochemical process. Unlike most chemical reactions, which produce heat as the main byproduct, these reactions also generate enough light to be detected, often visually and certainly using sensitive electronic photodetectors. This phenomenon is commonly observed in fireflies, although many other species are capable of producing light. Fluorescence is the production of a longer wavelength of light by a substance when it is excited by a shorter wavelength of light. This is commonly seen when ultraviolet light is used to produce a visible color that is not seen under white light.

Some bacterial species are bioluminescent; that is, they produce visible light. Bioluminescent bacteria contain *lux* genes, which encode the proteins needed for the bioluminescent reaction. The *lux* operon of *Vibrio fischeri*, which is used in our studies, is a complex pathway of five genes, *luxCDABE*, and efficient expression of all of these genes in the host is required for appropriate functioning of the bioreporter. Only two genes, *luxA* and *luxB*, encoding the heterodimeric luciferase enzyme, are needed for the actual bioluminescent reaction. The *luxCDE* genes have been implicated in the recycling of the required aldehyde substrate, so that a pool of substrate is continuously available. Several recent reviews describe the genetics and physiology of bacterial bioluminescence [12, 13] and the use of these fusions [1].

The advantages of bioluminescent bioreporters lie primarily in the relative ease of light measurement. Light can be measured accurately and with great sensitivity. Light radiates out in all directions from a point source, and so light detection can be performed in three dimensions, giving a more precise analysis of an object's position in space. It can be measured quickly (in real-time) and without perturbing or destroying the sample. For instance, the light detector can be introduced into the sample and left there for an extended period, or it can detect light that passes through the glass wall of a bioreactor vessel. In most environments, bioluminescence is a rare trait, and therefore a background problem is

unlikely. Light detection presents some difficulties in quantitation and in detection, since the amount of light produced per cell is small, but sensitive detectors are overcoming this problem.

Other useful bioreporter genes have been developed. The Green Fluorescent Protein (GFP) is a relatively new bioreporter that is developing into a versatile and valuable tool . GFP is superior to the lux genes in many applications, because the bioluminescent reaction requires substantial oxygen for correct functioning while GFP requires low oxygen concentrations. In addition, functioning of the GFP bioreporter is not dependent on an enzymatic reaction, nor does it require biochemical substrates. GFP is also extremely stable under most environmental conditions. Increased stability at high temperatures would be an asset for bioluminescent proteins, and substantial research effort has been directed towards this end.

The gene for Green Fluorescent Protein (GFP) is found in the jellyfish, *Aequorea victoria.* The GFP protein converts the blue bioluminescent light of the jellyfish to a green color; the advantage to the jellyfish of shifting the color from blue to green is not known. The GFP gene has been cloned and sequenced, and the protein has been extensively characterized [15, 16]. The protein that is synthesized from the GFP gene autocyclizes [5], producing a chromophore that is brightly fluorescent. When the GFP gene is expressed in a cell (either prokaryotic or eukaryotic), it fluoresces a bright green after cyclization of the chromophore [4]. The fluorescence makes the cell easy to detect using ultraviolet light (excitation: 395 nm, emission: 509 nm) and conventional light-gathering equipment.

As with the measurement of bioluminescence, fluorescence can be measured accurately and with great sensitivity. Detection is dependent on the ability of the researcher to expose the GFP molecule to the excitation wavelength, and this can be performed with flexible fiber optic cables that are introduced into a microbial ecosystem. Measurement is rapid, and there is no need to add any substrates or reagents. The problems of sample perturbation and destruction are therefore avoided.

Fluorescence of GFP is very bright, and individual bacterial cells can easily be seen by epifluorescent microscopy. GFP appears to be very slow in forming the chromophore (typically taking several hours), and the speed at which it forms seems to vary with different organisms and different growth conditions, although a comprehensive analysis of this phenomenon is lacking. The protein is extremely stable, and is largely unaffected by treatment with detergents, proteases, glutaraldehyde, or

organic solvents. It is also very stable over a pH range of 6 - 12, and in high (65 °C) temperatures. Its stability makes it ideal for some applications, such as for tagging bacteria for a transport experiment [3].

The intact GFP gene has been inserted into a derivative of Tn5, and therefore random mutations with GFP are possible[3]. This transposon, Tn5GFP1, can be introduced into a variety of Gram negative species using electroporation. This transposon is available to other researchers by contacting the corresponding author.

Mutations have been introduced into the GFP gene in order to produce fluorescent signals with altered properties. The Red Shifted - Green Fluorescent Protein (RS-GFP) was isolated in this manner [7]. The name refers to the shift of the excitation wavelength towards the red end of the spectrum. The protein fluoresces at approximately the same wavelength (the maximum is at 505 nm instead of 509 nm), but excites at 490 nm instead of 395 nm. This shift is expected to be helpful, since the 490 nm excitation wavelength is beyond the wavelengths of excitation for cellular proteins fluorescence (due to their aromatic amino acids). The RS-GFP gene is available on a plasmid, pTU58K (ClonTech, Palo Alto, CA). As this construct becomes more widely available, it will certainly be incorporated into broad host range plasmids for use as cloning vectors, and in transposons for mutagenesis methods. A mutant GFP developed by Heim et al. [9] results in the production of a blue color instead of green. It is anticipated that many more fluorescent protein genes will be isolated or created in the near future, such as the GFP gene from *Renilla reniformis* [11,17]. A spectrum of excitation and emission wavelengths might soon be available that would allow the use of several bioreporter genes in one species, or the use of bioreporters to distinguish individual species in a community [6].

2. Detection methods

The measurement of light can be accomplished by a variety of means. Visualization of bacterial colonies may be sufficient for some purposes, although the observer must avoid ambient light since the bioluminescent signal is dim compared to room light. Photographic film can be exposed to the light emitting from colonies, although this technique is usually cumbersome. Several types of electronic sensors

are suitable for the measurement of light. ATP photometers or luminometers, which are used for measurement of ATP concentrations by the luciferase assay, are common in laboratories. Liquid scintillation counters (LSC) are also common. A LSC must be very sensitive to detect photons resulting from radioactive decay, and so these make good photodetectors for bioluminescence, although the coincidence channel should be disconnected prior to use. The coincidence channel eliminates background during its measurement of radiation, but is a hindrance for bioluminescence work since light emanating from a single cell might not be detected by both photodetectors simultaneously. In bioluminescence work, a background sample can be tested, and therefore all light from samples should be measured. These methods are sensitive, but are not designed specifically for bioluminescent work. Accordingly, there are problems in introducing representative samples to the photodetectors, as well as incubation conditions for the samples. That is, the samples would have to fit inside ordinary scintillation vials, which might not provide adequate aeration or mixing.

Commercial photomultipliers (e.g. Oriel, Stratford, CT) are recommended for remote sampling of light, including bioreactors and soil microcosms. These usually include flexible fiber-optic cables which have a high efficiency of light transmittance, an important feature in measuring low amounts of light. For extremely low amounts of light, such as would be expected from single bacterial cells, charge-couple devices (ccd) can be used (e.g. Hamamatsu). The added sensitivity is reflected in the increased cost of this equipment, and few laboratories have access to one. A ccd can be used, however, to visualize signals that are seen through a microscope, and thus have the potential to describe the physiological response of single cells, although integration of weak signals can delay output for several minutes. Accordingly, samples that move or drift during the integration time will give a blurred image, if the image is detected at all. The lack of standardization is a major shortcoming of bioluminescent reporter work, and has its greatest impact on the quantification of results. A method to standardize photodetectors using a light-producing biochemical reaction has been described and this method should be applied more generally[14]. Calibration of a photodetector using a standard light source is possible, although the equipment is expensive and not generally available in laboratories.

Bacterial colonies expressing GFP can be easily detected upon exposure to a UV light; an inexpensive hand-held UV light will work well. Fluorescent bacteria can also be easily seen using epifluorescent microscopy. An appropriate filter set should be used; the filter for fluorescein detection has proved to be very useful for this purpose. A xenon or mercury lamp can be used as a source of UV excitation. Fluorescence spectrometry facilitates detection of GFP fluorescence. Fluorescent spectrometers vary in sensitivity and versatility, although in general they should be able to detect GFP expression in bacteria. Quantification of bacteria in the sample is possible when a standard is examined contemporaneously. Digital imaging spectroscopy is an excellent means of detecting and characterizing fluorescent signals, although the expense of the system makes it generally unavailable [8, 18].

3. Examination of samples ex situ

Some of the first practical (as opposed to pure research) uses of this technology was in the detection of specific chemicals after spillage at environmental sites. This type of work is exemplified by our work with the U.S. Army at their base on Kwajalein atoll [2, 10]. This base had a number of large fuel tanks that were leaking after many years of use, and the Army was concerned that the leakage was harming the environment. However, they did not know the extent of the contamination problem.

Samples were taken from the site and returned to the laboratory, where a test was designed for petroleum hydrocarbons. Since toluene and naphthalene are both components of petroleum hydrocarbons, bioreporters for both of these compounds were used to check soil samples for their presence. A simple test was designed, in which the bacteria were grown under defined conditions and then mixed in a slurry with the soil samples. Several samples could be tested at one time, and the entire test period was only about three hours. Results clearly showed which samples were contaminated. This was a significant finding, since the contamination was shown to be localized to the region surrounding the leaking tanks. This translated into great savings for the sponsor, since many cubic yards of soil would not need to be excavated and treated. The test was performed cheaply and with an accuracy that paralleled the best wet chemistry methods.

This technique is practical for the examination of other possible spill sites, such as leaking fuel tanks at service stations. Borehole samples are suitable for testing, and preparation of the samples is minimal. With the appropriate set of standards and controls, an approximation of the quantity of the contaminant can also be obtained. This information may be useful to demonstrate the extent of a contaminant plume, and how it changes temporally and spatially.

4. A dual bioreporter for on-line use

One of the problems in working with bioluminescence is that of normalization for cell number. That is, we are unable to tell from a given light source whether a few bacteria are creating a large quantity of light, or if many bacteria are creating a small amount of light that in the aggregate appears bright. To address this concern, we have incorporated the GFP bioreporter into a strain that detoxifies mercury, and which reports on this activity by the production of bioluminescence. The GFP fusion is constitutively expressed, and therefore a constant fluorescence is associated with each cell. From the quantification of the fluorescent signal we can determine how many cells are present in the field of view of the photodetector. The quantity of bioluminescence measured is then normalized for the cell number as determined by the fluorescence measurement.

This technique was initially attempted with biofilm growth on glass coupons (Khang et al., manuscript in preparation). The bacteria could be visualized using an epifluorescent microscope. The light measurement was obtained with a Hamamatsu VIM 3 system and Argus 50 control software. the results demonstrated that both signals could be detected under these conditions. The bioluminescence was induced with the addition of mercury at a concentration in the part-per-billion range, which also demonstrates the impressive sensitivity of these microorganisms for the detection of specific chemicals. A more sophisticated set of experiments involving this strain will incorporate extremely diverse microbial consortia (essentially three-dimensional communities) that may be important in ecosystem response to the introduction of toxic chemicals.

5. Field use of this technology

During the summer of 1996, the first field release of a genetically engineered microorganism for the purpose of bioremediation will take place on the Oak Ridge Reservation. This field release will actually take place in several large outdoor containers, called lysimeters, that will be filled with soil that has been contaminated with a mixture of polyaromatic hydrocarbons. Before the soil is loaded into the lysimeters, a culture of the bioreporter microbe HK44 will be added. This microorganism degrades naphthalene and similar aromatic compounds, and produces bioluminescence during this activity. The lysimeters will be instrumented with photomultipliers attached to fiber optic cables, which allow us to examine distant sites continuously over the course of the experiment.

One of the goals of this experiment is to determine which conditions are conducive to bioremediation activity. It is hoped that some sites will demonstrate bioluminescent activity (and hence biodegradation of the naphthalene) while other sites that are spatially close do not. by sampling these sites at the appropriate times we may discover which environmental parameters are critical in biodegradation processes. We will be able to take physical samples (soil and water) and examine them for a variety of characteristics, both microbial and physical. The result should be an unprecedented analysis of microbial activity in situ.

6. Summary

Bacteria are invaluable for a great many uses, such as bioremediation of hazardous wastes, production of valuable biochemicals, and recovery of minerals that are present in small concentrations. Understanding the processes by which bacteria work, particularly in microbial consortia and in situ, is a difficult task because of the complexity of the systems and the small size of the members of their communities. Study of these systems can be facilitated using bioreporter genes, particularly those genes that produce light as their response. Sensitive detection equipment can track these bacteria in situ and in small numbers, allowing a degree of resolution that is necessary to the understanding of microbial community dynamics.

These bioreporter bacteria have been used in laboratory scale experiments to track specific chemical compounds, and will soon be used in the first field release of a genetically engineered

microorganism for the purpose of bioremediation. Dual bioreporter bacteria are a new refinement in bioreporter technology that increase our ability to predict specific activities of microorganisms. As other bioreporter genes and gene fusions are developed, there is an increasing need to develop detection systems that are even more sensitive, and which can resolve activity on a cell by cell basis i.e. at the scale of micrometers. The succession fusion of these two technologies will allow real time, on-line measurement of individual bacteria, which will be an important factor in the development of new theories on bacterial behavior, growth, and development.

7. Acknowledgments

Based on work supported by the Office of Health and Environmental Research, U.S. Department of Energy. Oak Ridge National Laboratory is managed for the U.S. Department of Energy under contract DE-AC05-96OR22464 with Lockheed Martin Energy Systems, Inc. Publication no. XXXX , Environmental Sciences Division, ORNL.

8. References

1. Burlage, R.S. and C. Kuo. 1994. Living biosensors for the management and manipulation of microbial consortia. Ann. Rev. Microbiol. 48: 291-309.
2. Burlage, R.S., A.V. Palumbo, A. Heitzer, and G.S. Sayler. 1993. Bioluminescent reporter bacteria detect contaminants in soil samples. Appl. Biochem. Biotech. 45/46: 731-740.
3. Burlage, R.S., Z. Yang, and T. Mehlhorn. A Tn5 derivative labels bacteria with Green Fluorescent Protein for transport experiments. Gene 173: 53-58.
4. Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. Science 263: 802-805.
5. Cody, C.W., D.C. Prasher, W.M. Westler, F.G. Prendergast, and W.W. Ward. 1993. Chemical structure of the hexapeptide chromophore of the *Aequorea* green-fluorescent protein. Biochemistry 32: 1212-1218.

6. Crameri, A., E.A Whitehorn, E. Tate, and W.P.C. Stemmer. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nature Biotechnology 14: 315-319.

7. Delagrave, S., R.E. Hawtin, C.M. Silva, M.M. Yang, and D.C. Youvan. 1995. Red-shifted excitation mutants of the green fluorescent protein. Bio/Technology 13: 151-154.

8. Goldman, E.R. and D.C. Youvan. 1992. An algorithmically optimized combinatorial library screened by digital imaging spectroscopy. Bio/Technology 10: 1557-1561.

9. Heim, R., D.C. Prasher, and R.Y. Tsien. 1994. Wavelength mutations and posttranslational autoxidation of green fluorescent protein. Proc. Natl. Acad. Sci. USA 91: 12501-12504.

10. Heitzer, A., R.S. Burlage, and G.S. Sayler. 1992. lux gene bioreporters. in "Bioremediation of Petroleum Contaminated Soil on Kwajalein Island: Microbiological Characterization and Biotreatability Studies", H.I. Adler, R.L. Jolley, and T.L. Donaldson, eds.ORNL/TM-11925.

11. Lorenz, W.W., R.O. McCann, M. Longiaru, and M.J. Cormier. 1991. Isolation and expression of a complementary DNA encoding *Renilla reniformis* luciferase. Proc. Natl. Acad. Sci. USA 88: 4438-4442.

12. Meighen EA. 1991. Molecular biology of bacterial bioluminescence. Microbiol. Rev. 55:123-42.

13. Meighen, E.A. 1994. Genetics of bacterial bioluminescence. Ann. Rev. Genet. 28: 117-139.

14. O'Kane, D.J., M. Ahmad, I.B.C. Matheson, and J. Lee. 1986. Purification of bacterial luciferase by high-performance liquid chromatography. Meth. Enzymol. 133: 109-128.

15. Perozzo, M.A., K.B. Ward, R.B. Thompson, and W.W. Ward. 1988. X-ray diffraction and time-resolved fluorescence analyses of *Aequorea* green fluorescent protein crystals. J. Biol. Chem. 263: 7713-7716.

16. Prasher, D.C., V.K. Eckenrode, W.W. Ward, F.G. Prendergast, and M.J. Cormier. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111: 229-233.

17. San Pietro, R.M., F.G. Prendergast, and W.W. Ward. 1993. Sequence of chromogenic hexapeptide of *Renilla* green-fluorescent protein. Photochem. Photobiol. 57: 63S.

18. Youvan, D.C. 1994. Imaging sequence space. Nature 369: 79-80.