Visualizing gene expression in situ

Robert S. Burlage[1]

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

ABSTRACT

Visualizing bacterial cells and describing their responses to the environment are difficult tasks. Their small size is the chief reason for the difficulty, which means that we must often use many millions of cells in a sample in order to determine what the average response of the bacteria is. However, an average response can sometimes mask important events in bacterial physiology, which means that our understanding of these organisms will suffer. We have used a variety of instruments to visualize bacterial cells, all of which tell us something different about the sample. We use a fluorescence activated cell sorter to sort cells based on the fluorescence provided by bioreporter genes, and these can be used to select for particular genetic mutations. Cells can be visualized by epifluorescent microscopy, and sensitive photodetectors can be added that allow us to find a single bacterial cell that is fluorescent or bioluminescent. We have also used standard photomultipliers to examine cell aggregates as field bioreporter microorganisms. Examples of each of these instruments show how our understanding of bacterial physiology has changed with the technology.

Keywords: bacteria, bioreporter, bioluminescence, fluorescence, field release, microscopy.

1. INTRODUCTION

It has now become possible to examine the response of a single bacterial cell to changes in its environment. Microprobes have been designed that can be inserted in the midst of a group of bacteria or near a single cell. In this manner oxygen concentration and pH can be monitored on a minute scale. However, it is also important to detect the physiological processes that occur inside of a single cell. Such cells may ultimately be used to construct biosensors, which are fusions between electronics and the life sciences. For such a device an accurate assessment of the cell's requirements and normal responses is needed. While eukaryotic cells have been proposed for biosensors, prokaryotic cells should also be considered because of their relative durability, their unique biochemical pathways, and their size.

Bacteria are very sensitive to their surroundings, and can respond relatively quickly when they contact appropriate nutrient streams. They can respond to chemical concentrations over a wide range, and

[1]For further information, please contact the author at tel: 423-574-7321; fax: 423-576-8543; e-mail: RVX@ORNL.GOV

DISCLAIMER

are usually tolerant of suboptimal conditions such as might be caused by changes in pH or oxygen concentration. For many years this laboratory has been studying the response of bacterial strains to environmental changes with the aim of determining the conditions under which certain processes are favored, such as bioremediation of hazardous wastes. In the course of this study several unique opportunities have arisen that have enabled the exploitation of bacteria for unanticipated purposes.

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. These bioreporter microorganisms are described, along with the technology for detecting them and the projects which are benefiting from their application.

2. DESCRIPTION OF THE TECHNOLOGY

1. Genetic engineering

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, 19].

3. CASE STUDIES

Case 1. Field release for bioremediation.

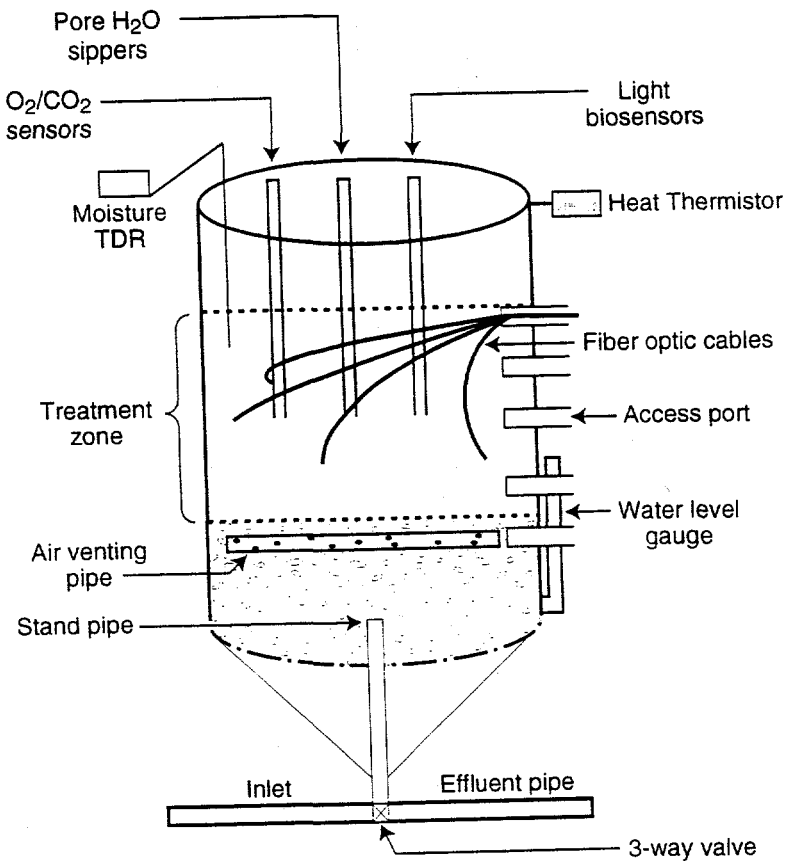
During the summer of 1996, the first field release of a genetically engineered microorganism for the study of bioremediation took place on the Oak Ridge Reservation. This field release was in several large outdoor containers, known as lysimeters, that were filled with soil that had been contaminated with a mixture of polyaromatic hydrocarbons (naphthalene, anthracene, and phenanthrene). Before the soil was loaded into the lysimeters, a culture of the bioreporter microbe HK44 was added. This microorganism degrades naphthalene and similar aromatic compounds, and produces bioluminescence during this activity. The lysimeters were instrumented with photomultipliers attached to fiber optic cables, which allow us to examine distant sites continuously over the course of the experiment. A schematic of the instrumentation used in these lysimeters is shown in Figure 1. We were also able to take physical samples (soil and water) and examine them for a variety of characteristics, both microbial and physical. The result was an unprecedented analysis of microbial activity in situ[18].

This project has been very successful in following bioremediation through the course of many months and many different treatments. It was found that the major limiting factor in bioremediation is oxygen; when air was sparged into the system the degradation of naphthalene increased dramatically, as did the light production observed by the photosensitive recorders. These results provide a key to effective bioremediation in actual field sites. During the study the bacteria living in the soil were difficult to see since they were present at a concentration of approximately 10^5 bacteria per gram of soil, which is rather low. However, use of biosensors that incorporate the bioluminescent bacteria into the fiber optic probe tip clearly showed when conditions were optimal for degradation to take place.

Persistence of the bacteria at the field site was also easy to measure because the bioluminescence provided a handy screen for the bacterial species of interest. Bioluminescent bacteria that respond to naphthalene are virtually unknown in natural ecosystems, and therefore there is no background to consider in such assays. A selection for the bacteria on antibiotic-containing agar plates was followed by screening for light production by the colonies produced on those plates. This method was also used to find any evidence of genetic transfer between species in the wild, since bacteria that pick up the recombinant genetic constructions would likely retain the antibiotic resistance and might also show bioluminescent activity. After careful study of many samples, we have been unable to find a single instance of genetic transfer. Such transfers in the wild must be exceedingly rare events.

ORNL-DWG 95M-7148

Cross Section of Lysimeter Showing Positions of Equipment and Sampling Ports



Case 2. Gene expression in a biofilm.

This technique was initially attempted with biofilm growth on glass coupons. 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 has incorporated extremely diverse microbial consortia (essentially three-dimensional communities) that may be important in ecosystem response to the introduction of toxic chemicals.

A single strain of genetically engineered bioreporter bacteria has been used to detect activity under biofilm conditions. The bacteria are identifiable in the mixed community due to their fluorescence, and the activity of select genes is found by their bioluminescence. This has been shown using bioreporters of toluene and xylene. For these experiments a laser confocal microscope and a powerful photomultiplier have been arranged in tandem so that the two signals can be correlated. Gene expression under these circumstances has already shown some unexpected results, suggesting that bacteria growing planktonically are far different from those grown attached to a surface. The identification of genes that are involved in this new mode of gene expression will be the subject of future investigation.

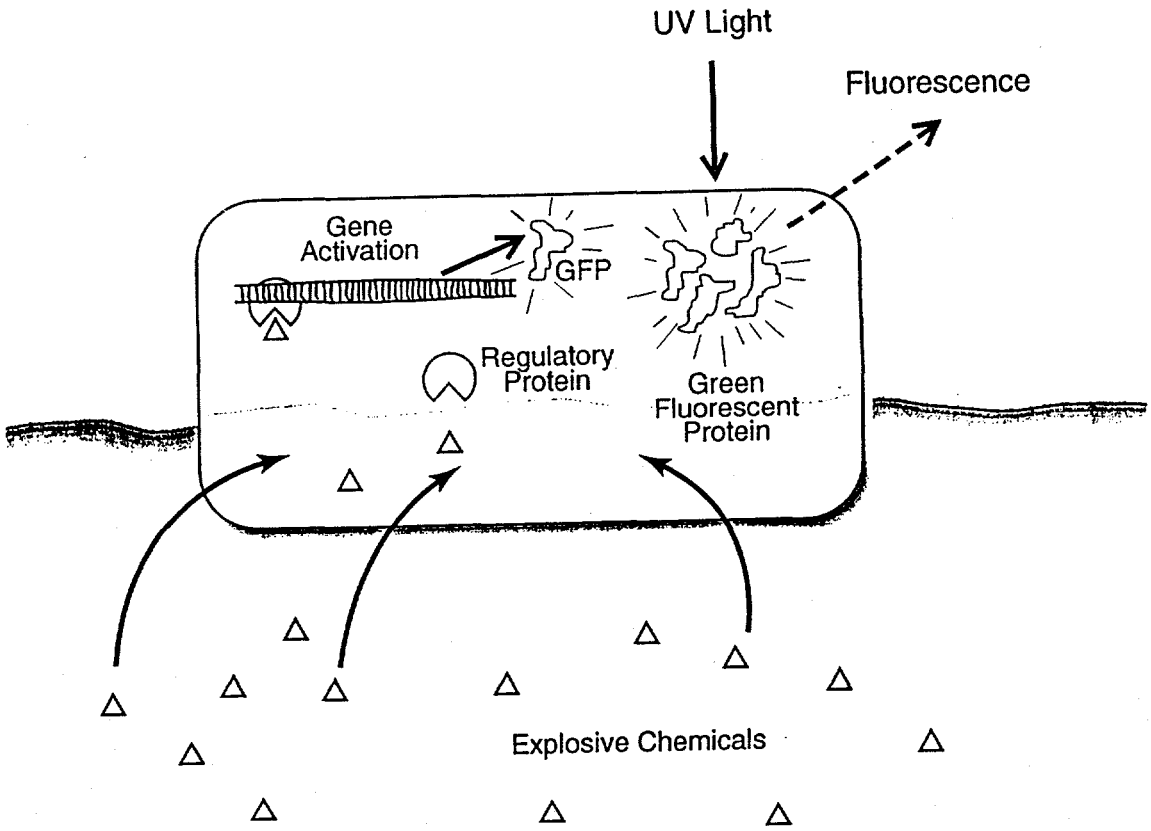
Case 3. Detection of unexploded ordnance in the field.

In many areas of the world, one of the major societal problems is the presence of landmines. These devices are often left over from armed conflicts, and in many cases their whereabouts are undocumented. This means that large areas within a country are inaccessible, and this may limit the amount of arable land available to a country. Obviously this may lead to a shortage of food in that country, and so the problem is compounded.

Methods to destroy landmines in the ground are well established and safe, but the limiting factor is discovering where the mines are located. At the present time the best method for locating mines is the use of dogs that can detect explosives by their sense of smell. From this observation we know that small amounts of explosives leak from the mines and are found in the vapor phase near the mine. This makes the use of bacteria ideal for detection, since they are responsive to small concentrations of chemicals. In some studies the concentrations of explosives at landmine sites has been measured in the part-per-billion to part-per-million range.

A pair of bacterial strains was developed that can be used for field detection of trinitrotoluene (TNT), currently the most common explosive found in landmines. One of these strains is bioluminescent and the other is fluorescent in the presence of TNT (Figure 2). The fluorescent strain has unique advantages that make it the choice for field use, although in the first field demonstration both strains were tested. The field demonstration was performed at a site in South Carolina that had been seeded with explosive devices in a known area. The bacteria were sprayed over the surface of the field in the early evening and were checked for activity after approximately four hours.

ORNL 96M-1234



The bioluminescent strain, as expected, was unsuitable for field use. The signal from the bacteria was relatively weak and the interference with ambient light sources, even in the middle of the night, was significant. However, the fluorescent strain was very effective. All of the targets were discovered using this strain, which was detected by two teams of fluorescence monitors. In the simplest application, the ground was illuminated with an ultraviolet light while the observer scanned the ground through a notch filter. This was sufficient for detection of the signature fluorescence. The other team employed a strobed ultraviolet light and a more sophisticated electronic detection scheme. This apparatus should be suitable for detection of the signal under daylight conditions, and will be tested in this manner in the near future.

4. 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 increases 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.

5. ACKNOWLEDGEMENTS

Research sponsored by the Joint Bioremediation Program of the Office of Biological and Environmental Research, U.S. Department of Energy. Oak Ridge National Laboratory is managed by Lockheed Martin Energy Research Corp. for the U.S. Department of Energy under contract number DE-AC05-96OR22464.

5. 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. Sayler, G.S., C. Cox, R.S. Burlage, and U. Matrabutham. Releasing a genetically modified microorganisms for bioremediation: one-year field experience. Environ. Sci. Technol. (submitted)
19. Youvan, D.C. 1994. Imaging sequence space. Nature 369: 79-80.