LA-UR-96- 1928

Title:

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Submitted to:

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Development and Testing of Biosensors that Quantitatively and Specifically Detect Organic Contaminants

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Abstract

This is the final report of a two-year Laboratory-Directed Research and Development (LDRD) project at the Los Alamos National Laboratory (LANL). The project sought to develop a more sensitive and less expensive method of detecting organic contaminants. Assaying complex environmental samples for organic contaminant content is costly and labor intensive. This often limits extensive testing. Sensitive microbial biosensors that detect specific organic contaminants in complex waste mixtures without prior separation from other waste components have been developed. Some soil microbes degrade organic compounds that contaminate the environment. These bacteria sense minute quantities of particular organic compounds then respond by activating genes encoding enzymes that degrade these molecules. Genetic manipulation of these gene regulatory processes has been employed to develop unique biosensors that detect specific organic compounds using standard biochemical assays. Such biosensors allow rapid, sensitive testing of environmental samples for selected organic contaminants. The cost of biosensor assays is at least 100-fold less than present methods, allowing more rapid and extensive testing and site characterization.

1. Background and Research Objectives

Application of different environmental remediation technologies is dependent on the type and extent of contamination. Removal or treatment of surface deposits may suffice in some cases while flushing an aquifer may be required in others. Site restoration has not succeeded in some notable cases despite expenditure of billions of dollars. Before remediation can proceed, a site must first be characterized to determine types and concentrations of contaminants. Assaying for organic compounds in complex environmental samples is a costly (\$200 - \$2000/assay) and labor intensive process. This cost often limits extensive testing.

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Without spatially and temporally distributed, replicated assays, the true extent of a contaminated site remains unknown. Under-estimating the extent of contamination results in ineffective remediation while over-estimation increases remediation costs.

Bacteria that degrade organic environmental contaminants are widespread. These microbes possess enzymatic pathways that degrade selected organic molecules, often providing the organism with carbon and energy sources. The enzymes that catabolize these compounds are encoded by one or more genes. These genes are often tightly regulated so that, in the absence of the organic substrate, they are not expressed. The bacterial regulatory systems that control these processes sense minute quantities of particular organic compounds in their environment, then respond by activating the genes encoding the enzymes that degrade these molecules. In some cases, the regulatory mechanisms have been well studied, their components characterized and their mechanism of action understood.

The object of this research was to take advantage of the sensitivity and specificity of biological systems to develop biosensors that can detect targeted compounds in complex mixtures without first purifying these contaminants. We proposed to develop biosensors that are very sensitive to and specific for particular organic molecules. These sensors would take advantage of bacterial regulatory systems that sense minute quantities of particular organic compounds in their environment then respond by activating genes encoding enzymes that degrade these molecules. Many aromatic and halogenated organic chemicals (e.g., toluene, benzoate, naphthalene, TCE, chloroform, TNT) are degraded by different bacteria. We proposed to genetically engineering the promoters (the switches that regulate activity of the above genes) and sensory proteins that regulate the enzymes that destroy these compounds to act as specific biological monitors of these contaminants. By genetically manipulating these regulatory processes we expected it would be possible to develop unique biomonitors that specifically detect certain organic compounds. Moreover, we also expected that modification of these processes might produce biosensors that respond to entire classes of compounds, allowing rapid, sensitive, inexpensive testing of complex environmental samples.

2. Importance to LANL's Science and Technology Base and National R&D Needs

This research addressed two different Laboratory core competencies. Development of processes that detect environmental contaminants addresses issues related to earth and environmental systems while development and modification of the biosensors themselves increases competency and expertise in biosciences and biotechnology. Biosensors will allow less expensive environmental site characterization. This addresses a serious national need. Availability of inexpensive sensors will allow detailed site characterization before and during

remediation processes. These sensors will provide the means to test developing remediation technologies thoroughly while providing rapid methods to screen environmental sites suspected of containing harmful contaminants. Developing this technology required skills in molecular biology to construct and test biosensors and expertise in biochemistry to understand the biochemical processes. Optimization and modification of biosensors required expertise in structural biology to understand protein-organic compound interactions and binding of regulatory proteins to specific DNA sequences. A thorough investigation of these processes provided information to develop new classes of biosensors while developing a better understanding of protein structure: function relationships.

Completion of these experiments provided fundamental new information that is critical for two new Laboratory projects. The first uses the biosensors we developed as a resource to produce a new generation of sensors. The program applies new molecular technologies to enhance the sensitivity and specificity of the current biosensors while developing a more general method of biosensor development to detect compounds not easily monitored in the environment. At the same time, it provides data to address relationships between protein structure and function, particularly with respect to DNA binding. The second uses the information generated by the biosensor study to understand regulation of modified genes placed into soil microbes. This is an important part of science-based bioremediation core competency development at Los Alamos.

3. Scientific Approach and Results to Date

We have designed and constructed a biomonitor that detects benzoic acid and used this to develop a rapid, inexpensive bioassay for benzoic acid. This assay has now been evaluated using environmental samples. The benzoic acid biomonitor was constructed by genetically engineering *E. coli* with a plasmid containing the regulatory gene *xylS*, a transcriptional promoter sequence *Pmeta*, the transcriptional terminator sequence *rrnB*, the reporter gene encoding luciferase, and a selectable marker gene for ampicillin resistance. In this assay, the regulatory gene produces the protein *xylS* which binds to the ligand benzoic acid in a concentration-dependent manner (Figure 1). The protein-ligand complex then binds to the transcriptional promoter *Pmeta*, activating transcription of the luciferase reporter gene. Luciferase activity is detected using an innovative one-step lysis reagent followed by addition of enzyme substrates. Luciferase generates light that is measured either electronically (photo multiplier tube based detection) or with photo-sensitive emulsions (Polaroid film) (Figure 2). The assay is quantitative between 0.75 and 1,000 ppm of benzoic acid. It is highly reproducible and equivalent detection is achieved with either electronic or emulsion based systems. The assay involves the use of live bacterial cultures for the induction of the reporter

gene. We obtained similar results using recently cultured cells, frozen glycerol cells, or lyophilized cells as starting material. Lyophilized cultures offer several advantages: easily transported to the field sites, dependable maintenance of the plasmid construction, and long shelf life.

A field assay for benzoic acid was developed to allow high throughput of samples with minimal equipment requirements. We have developed and constructed a field luminometer based on Polaroid film detection. This "camera" is designed around a Polaroid camera film back (\$90 each), a black acrylic box (ca. \$25 each), and a modified 80-well microtiter dish (ca. \$5 each). This device is about 8 x 5 x 5 inches in size and is capable of performing greater than one thousand assays per hour. Field sample preparation procedures have been developed that range from merely filtering the sample through 0.5-micron filters (30 seconds per sample; cost ca. \$2 per sample) to concentration of large volumes using C18 cartridges (ca. \$4 each). Timed exposures from the assay can be scanned into a computer and the intensity of the exposure measured using standard analysis software (SigmaScan, Jandel Inc). Analysis can be accomplished in the field with portable computer equipment or after transporting the photographic films to the laboratory. Analysis by computer provides high volume capacity and great accuracy, but a small number of samples can be evaluated "by eye" in a semi-quantitative manner. This is an extremely inexpensive system that is adaptable to any biomonitor assay that relies on light generation.

The biomonitor and field kit were evaluated at the Mojave Generating Station in southern Nevada. This is a coal-fired electrical generating station that receives its coal in an aqueous slurry pipeline. The coal is removed from the water prior to burning. The water is heavily contaminated with aromatic organic compounds that have leached from the coal during transport. Among these is benzoic acid and related compounds. We have collected water samples from different processing steps and determined the benzoic acid content in the field using the biomonitor field kit. We have compared these results to laboratory experiments using the biomonitor and a standard microtiter luminometer reader; and to standard analytical chemical procedures using gas chromatography-mass spectroscopy. We always include concentration standards and "spiked" environmental samples to determine whether other contaminants in the samples are inhibiting the assay (Figure 2). We detected ca. 5 ppm benzoic acid in samples collected from several early steps in the water purification process. Later steps did not appear to contain detectable benzoic acid. Biomonitor assay results were confirmed using the standard analytical chemical techniques. These results demonstrate that the benzoic acid biomonitor is "field-ready" and suitable for determining benzoic acid in environmental samples.

We have completed and laboratory tested a second biosensor that detects toluene. This biomonitor is much more sensitive than the first, sensing toluene to concentrations below 70 ppb (Figure 3). It is genetically quite similar to the benzoic acid biomonitor except that the sensing protein (*xylR*) and the *Pupper* gene promoter sequence respond specifically to toluene. This microbial sensor is designed to function with exactly the same hardware and reagents as the benzoic acid biosensor. We are currently modifying the *xylR* gene to further increase the sensitivity of the system and to specifically detect a different contaminant, TCE.

Additional environmental chemicals can be quantitatively detected by biomonitor assays if bacterial systems metabolizing these chemicals can be identified. We are focusing on the chemical classes involving phosphonates. This is a central chemical moiety in many pesticide and neurotoxin contaminants. The toxicity of these compounds is so great that it precludes their use in routine laboratory procedures. Instead, we have chosen a non-toxic phosphonate herbicide, glyphosate. This chemical is heavily used in agriculture and certain microbes metabolize it as a phosphate source. We obtained soil from a nursery in Iowa exposed to glyphosate for nearly a decade. An enrichment procedure was developed where the bacterial microflora was grown in minimal salt media lacking a phosphate source with the exception of glyphosate. At this point we have 10 unique types. These include both gram positive (*Arthrobacter*) and gram negative (*Rhizobium*) bacteria that have unique capabilities to grow on different phosphonate compounds. We are currently testing four different plasmid vectors for compatibility with these bacterial isolates. The useful vectors will be modified for "promoter trapping" experiments to develop the next generation of environmental biomonitors.

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Figure 1. Schematic of biosensor design and function. The *xylS* gene produced *xylS* protein (1) that binds to benzoic acid entering the cells (2). This complex binds to the promoter (3) activating the luciferase gene (4). Luciferase produces light (5) when provided with the appropriate reagents.

Figure 2. Photograph of bioluminescence induced by exposure waste samples from the Mojave Generating Station. Samples from different waste streams were tested using the benzoic acid bioluminescent to determine the amount of this compound in these solutions. S, CE, CL, P2, SER, FC and LC are different environmental samples. Different dilutions of each sample were assayed. High concentrations of some samples inhibited the reaction (LC). Benzoic acid standards were included (far right two lanes).

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Toluene Induction of Bacterial Biosensor

Figure 3. Induction of the toluene biomonitor by different toluene concentrations. Toluene biomonitor stocks frozen in glycerol were thawed, exposed to different toluene concentrations, then assayed for luciferase activity. Each plotted value represents three data points, demonstrating the reproducibility of the assay.