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Releasing a Genetically Engineered Microorganism for Bioremediation

Gary Sayler· Robert Burlage*· Chris Cox**· David Nivens· Steven Ripp · Yeonghee Ahn · Jim Easter· Claudia Wrner· John Jarrell

Center for Environmental Biotechnology, Department of Microbiology
**Department of Civil and Environmental Engineering, University of Tennessee
*Environmental Sciences Division, Oak Ridge National Lab. Oak Ridge

ABSTRACT

A field study was performed to test effectiveness of a bioluminescent genetically engineered microorganism (GEM) for bioremediation process monitoring and control. The study employed *Pseudomonas fluorescens* HK44 that was the first strain approved for field application in the U.S. for bioremediation purposes. HK44 contains *lux* gene fused within a naphthalene degradative pathway, allowing this GEM to bioluminesce as it degrades naphthalene as well as substituted naphthalenes and other polycyclic aromatic hydrocarbons (PAHs). Results showed that HK44 was maintained in both PAH-contaminated and uncontaminated soils even 660 days after inoculation. HK44 was able to produce bioluminescence in response to PAHs in soil. Although effectiveness of chemical remediation was not assessed due to heterogeneous distribution of contaminants, decreased concentration of naphthalene was shown in the soils. Taken together, HK44 was useful for in situ bioremediation process monitoring and control. This work is so far the only field release of a GEM for bioremediation purposes.

Key words: genetically engineered microorganism, polycyclic aromatic hydrocarbon, bioremediation, and lysimeter

I. Introduction

Genetically engineered microorganisms (GEMs) have been designed to

exhibit enhanced abilities to degrade or monitor many chemical contaminants. Laboratory-based experimental data show that GEMs have potential for bioremediation of contaminated environments (10). Long-term field release studies are necessary to address the competence of GEMs in bioremediation. Field studies can provide the information about the overall effectiveness and risk associated with GEM introduction into natural ecosystems. However few examples of such studies have been reported.

Polycyclic aromatic hydrocarbons (PAHs) are a class of priority pollutants that consist of two or more fused-benzene rings. They are ubiquitous and mainly produced from incomplete combustion of organic materials. Major sources are anthropogenic such as fossil fuel combustion, automobile exhaust, and waste incineration. Because of their persistence in the environment and their toxicity, remediation of PAH-contaminated environments is important. As a model compound for PAHs, naphthalene degradation is the best studied of the PAHs in microbial systems. The bacterial degradation of naphthalene has been well characterized for the naphthalene catabolic enzyme system encoded by the plasmid NAH7 of *Pseudomonas putida* PpG7. Many PAH-contaminated soils contained PAH-degrading bacteria with NAH7-like genotype (1).

A bioluminescent reporter strain *P. fluorescens* HK44 (Nah^+ Sal^+ Lux^+ Tet^+) was the test GEM for the project. HK44 contains a naphthalene-degradative plasmid (pKA21) that is very similar to archetypal naphthalene-catabolic plasmid NAH7. Plasmid pKA21 contains a promoterless *luxCDABE* gene cassette inserted into the naphthalene-degradative pathway (6). In the presence of naphthalene, substituted naphthalenes and other PAHs such as phenanthrene, HK44 produces light that can be monitored. Previous laboratory studies showed that HK44 exhibited a positive correlation between naphthalene degradation and bioluminescence (5).

The U.S. Environmental Protection Agency (EPA) under authority of the Toxic Substance Control Act (TSCA) assumes regulatory oversight of large scale and commercial use of a GEM for field release in bioremediation of hazardous chemicals (3, 4). To propose release of a GEM in the environment, a premanufacture notification (PMN) is submitted to the EPA and regulatory review process is initiated (12). As results of EPA TSCA review on the PMN

for HK44, the Univ. of Tennessee received an EPA consent order to allow field release of the GEM for bioremediation.

Under the first U.S. EPA sanction, the University of Tennessee in collaboration with Oak Ridge National Laboratory (ORNL) performed field release of a GEM for bioremediation purposes. The overall purpose of the project was to investigate the usefulness of GEMs for in situ PAH bioremediation. The specific goals of the investigation include 1) testing a GEM to monitor and control bioremediation, and 2) testing survival and maintenance of a GEM in a bioremediation.

II. Materials and methods

1. Lysimeter design

HK44 was released into ORNL field lysimeter facility on October 30, 1996 (8, 11). The lysimeters were originally constructed for studying leachate from radioactive wastes but never used for the purpose. They were modified for this study and served as a controllable field site (2).

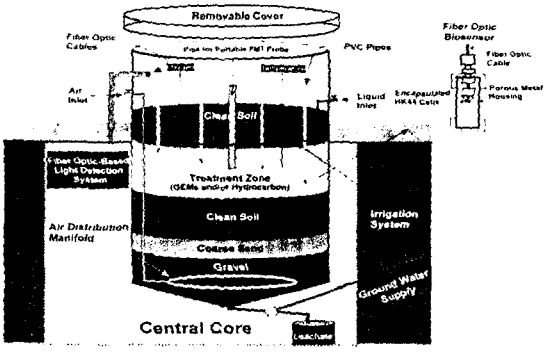

Fig. 1. Schematic of a lysimeter.

HK44 was introduced into 6 lysimeters containing soil with or without PAHs (Fig. 1); lysimeters 1, 2, and 4 received PAH-contaminated soil inoculated with HK44, lysimeters 3 and 5 received uncontaminated soil inoculated with HK44, and lysimeter 6 received PAH-contaminated soil only.

Each lysimeter was 4 m deep by 2.5 m in diameter constructed of galvanized

steel pipe buried 3 m below ground surface. Various detectors were buried in each lysimeter to measure temperature, moisture, CO_2, and oxygen. An air distribution device
was set up in the bottom of each lysimeter to provide oxygen when needed. A plumbing system was used to add water and nutrients when needed.

2. In situ detection of bioluminescence

Light sensing instrument consisted of buried optic cables and biosensors. Fiber optic cables were buried at different depths within the lysimeters to measure bioluminescence directly from HK44 in the soil (8, 11). Biosensors were vertically dropped down into PVC pipes installed at various depths with in the lysimeters. Light emitted from HK44 was transferred through the cable to a liquid light guide. Photomultiplier tube (PMT) detected photons collected by the light guide and generated electronic pulses. Pulses from the PMT were amplified and the number of photons per second was determined and recorded by a computer. In addition to buried fiber optic cables, a portable PMT system was used to detect bioluminescence directly from soil.

3. Microbiological and chemical analyses

Soil samples were taken to determine populations of HK44 and total indigenous bacteria and concentration of PAHs. Bacterial numbers were determined by plate counts and colony hybridization (9, 11).

A solvent extraction method was used to obtain PAHs in soil samples and PAH concentrations were analyzed with a gas chromatograph-mass spectrometer as described (2).

III. Results and discussion

1. Survival of HK44

HK44 could survive in both PAH-contaminated and uncontaminated soils (Fig. 2 & 3). Selective plate counting showed that initial concentrations of presumptive HK44 population were 1.5 x 10^6 cfu/g soil in PAH-contaminated lysimeters and 1.7 x 10^6 cfu/g soil in uncontaminated ones. Within 2 weeks, HK44 decreased to 2.4 x 10^5 cfu/g soil in PAH-contaminated lysimeters and 3.4 x 10^5 cfu/g soil in uncontaminated lysimeters. Lysimeters 1, 2, 4, and 6 received inorganic nutrients and supplementary

PAHs dissolved in Exxon Univolt 60 transformer oil on day 135. This treatment resulted in increase in presumptive HK44 numbers to 3.7 x 10^5 cfu/g soil on day 154 from 4.0 x 10^4 cfu/g soil on day 117. This growth produced depleted oxygen in soil and populations started to decline. Aeration system was operated to increase and maintain oxygen level to approximately 20% since then. KHK44 was recoverable from the lysimeter soil 660 days after inoculation. This suggested that HK44 was able to compete under real environmental conditions.

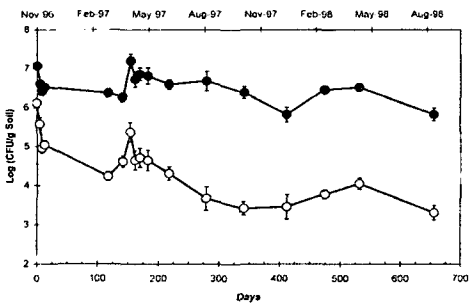

Fig. 2. Heterotrophic (•) and presumptive HK44 (o) population dynamics in the PAH-contaminated lysimeters 1, 2, and 4.

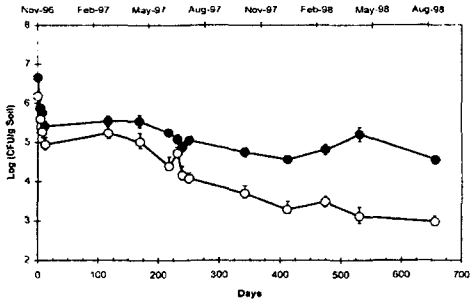

Fig. 3. Heterotrophic (•) and presumptive HK44 (o) population dynamics in the uncontaminated lysimeters 3 and 5.

To verify true HK44 colonies, colony hybridization using a *luxA* gene probe was performed on presumptive HK44 colonies on selective plates. Similar values were shown between colony hybridizations and selective plate counts in both contaminated and uncontaminated soils (Fig. 4). However after day 400, the percent of *luxA*-positive decreased in contaminated lysimeters, suggesting HK44 populations were decreasing with time. Fig. 5 shows HK44 population dynamics as determined by colony hybridization.

Population dynamics of heterotrophic bacteria was also determined by the plate counts using a nonselective medium (Fig. 2 & 3). Heterotrophic microbial populations decreased in all lysimeters after the inoculation of HK44. The addition of nutrient on day 135 also resulted in increase of heterotrophic populations. However they remained constant with time.

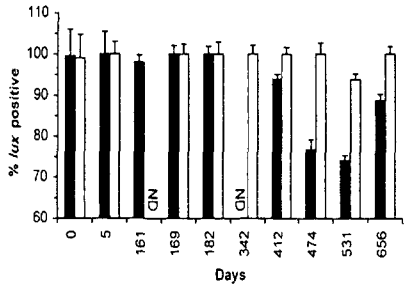

Fig. 4. Colony hybridization using a *luxA*-gene probe. No luxA-positive colonies were detected in lysimeter 6. ■, PAH-contaminated lysimeters; □, uncontaminated lysimeters; ND, not determined

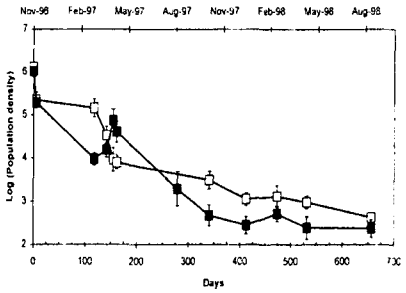

Fig. 5. HK44 population dynamics in the PAH-contaminated lysimeters (■) and uncontaminated lysimeters (□) as determined by colony hybridization.

2. Analysis of contaminants

Initial concentrations of naphthalene in PAH-contaminated soils were not detectable because of loss during the aging process of the soil before loading of the lysimeters. Fig. 6 shows initial naphthalene concentrations analyzed within 1 month after the supplementary PAH addition on day 135 and its final concentrations on day 474. GC/MS analysis of soil samples showed that naphthalene or total petroleum hydrocarbon concentrations were distributed heterogeneously throughout the soil.

Although effectiveness of chemical remediation was not assessed due to heterogeneous distribution of contaminants, decreased concentration of naphthalene was shown in the soils. Control lysimeter 6 also showed decreased concentration of PAHs without HK44, suggesting natural attenuation by other PAH degraders, and/or abiotic processes such as volatilization also affected PAH concentrations. Some indigenous bacteria growing on naphthalene as a carbon and energy source were isolated from the PAH-contaminated lysimeters.

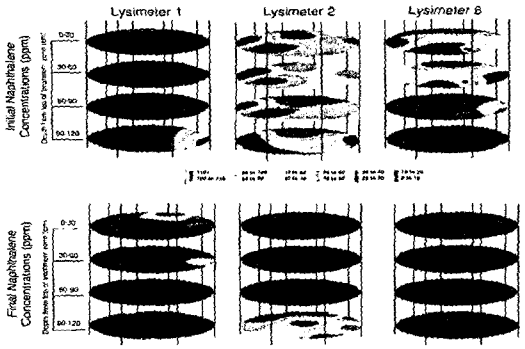

Fig. 6. Naphthalene concentrations in PAH-contaminated lysimeters. Initial concentrations were measured from 8 soil-cores taken within 1 month after addition of supplementary PAHs on day 135. Final concentrations were derived from a single sampling of 9 cores taken on day 474. The top 3 sections are the treatment zone while the bottom one is a soil layer just below the treatment zone.

3. Bioluminescence from HK44

HK44 was able to produce bioluminescence in response to PAHs in soil. Light was detected from HK44 in PAH-contaminated soil as well as HK44 immobilized in biosensor devices that specifically responded to volatile PAHs (Fig. 7 & 8).

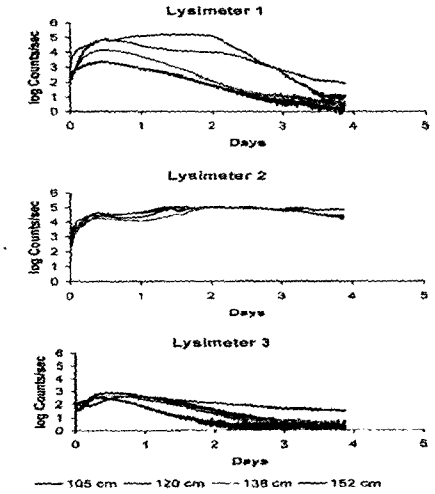

Fig. 7. Biosensor response to vapor phase PAHs. Four biosensors were located at various depths in lysimeters. Day 0 corresponds to day 169 of experiment. Depths start from soil surface.

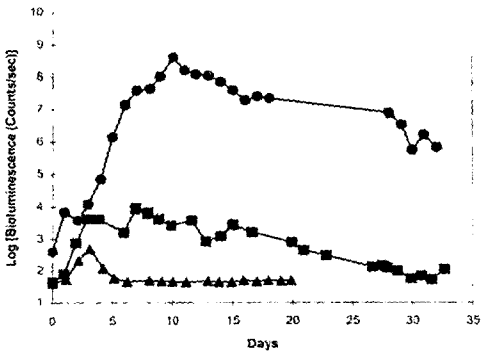

Fig. 8. Bioluminescence monitoring directly from PAH-contaminated lysimeter 4 soil using the portable photomultiplier tube. The response was obtained after addition of inorganic nutrient medium and naphthalene to soil beneath Plexiglas windows (■). ●, Bioluminescence in the absence of Plexiglas windows; ▲, bioluminescence in response to the addition of inorganic nutrients alone. Day 0 corresponds to day 444 of experiment.

Biosensors produced light signals as high as 166,000 counts/s. The signal gradually decreased to baseline after approximately 5 days due to call death caused by depletion of nutrients and water. In spite of heterogeneous distribution of PAHs in soils, all biosensors showed similar light response. This suggested that aeration system might have distributed volatile naphthalene throughout the soil bed.

The buried fiber optic cables were ineffective at detecting bioluminescence from HK44 population and/or activity at the level shown in this experiment. Therefore, a more sensitive portable PMT device was designed during this study and used on day 444. When localized areas of soil were challenged with inorganic nutrient medium and naphthalene, bioluminescence was initiated within a day and persisted up to 28 days; bioluminescence at an average maximum of 4,300 counts/s with a mean duration of 13 days (8, 11). Presence of Plexiglas windows reduced bioluminescent response of HK44, demonstrating oxygen was a limiting factor. Direct online detection of bioluminescence provided a continuous, real-time monitoring of the bioremediation process and contaminant bioavailability.

When GEMs are used, a special concern exists due to risk associated with the dissemination of engineered genotypes to indigenous populations in ecosystems. The use of large-scale lysimeter facility provides an adequate environment closest to actual environment without actual release of GEM into the environment. The

lysimeters serve as semi-contained environment with safety backup to prevent accidental GEM discharges. This field study showed that bioluminescence-based GEM HK44 could serve as a tool for monitoring and controlling bioremediation process. Future field application of GEMs in bioremediation may be limited by U.S. EPA's risk-based regulation on GEM in bioremediation (7). However GEM can be used in contained reactor system for soil bioremediation or wastewater treatment. It has been generally realized that clean up is more expensive than pollution abatement that is more expensive than pollution prevention.

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