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This research focuses on developing new molecular knowldege and techniques to develop a greater understanding of microbial degradation processes in the environment. A major goal of this research is to create a molecular strategy for quantitative biodegradation process monitoring and control in the environment and to predict success and failure patterns impinging on biodegradation of hazardous waste materials.

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Annual Progress Report (Jan. - Dec. 1992)

Molecular Ecology of Bacterial Population in Environmental Hazardous Chemical Control

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Background

This research focuses on developing new molecular knowledge and techniques to develop a greater understanding of microbial degradation processes in the environment. A major goal of this research is to create a molecular strategy for quantitative biodegradation process monitoring and control in the environment and to predict success and failure patterns impinging on biodegradation of hazardous waste materials. Among the major outcomes of the current year are:

(1) Creation of a toluene dioxygenase bioluminescent (*tod-lux*) reporter system in *Pseudomonas putida* F1 to measure toluene driven co-metabolic oxidation of TCE.

(2) Demonstration of a new pathway for aerobic biodegradation of DDT, mediated by *Alcaligenes eutrophus* strain A5 previously shown competent for biodegradation of chlorobiphenyl congeners.

(3) Confirmation that environmental strains containing a known plasmid encoded, naphthalene catabolic pathway uses this pathway to simultaneously degrade other polyaromatic hydrocarbons such as anthracene and phenanthrene.

The specific objectives of this research project are described as follows:

1) Development and application of molecular microbial ecology techniques for environmental research use.
   a) Analysis of the use of bioluminescent reporter strains for measuring biodegradation of

environmental pollutants.

b) Application of DNA probe technique for measuring catabolic gene(s) frequency in the environment.

2) Validation and standardization the *nah-lux* bioluminescent model system as a tool to detect organic contaminates in the environment.

3) Exploitation of the *nah-lux* bioluminescent reporter systems for measuring organic pollutant bioavailability and biodegradation, and ecological and environmental factors involved in controlling microbial degradation processes.

Research Progress

The current research is multifaceted and includes fundamental studies on metabolism of chlorinated organics (TCE, chlorobiphenyl and DDT), monoaromatics (toluene), and polyaromatic hydrocarbons such as naphthalene, anthracene, phenanthrene and higher molecular weight PAH. This research is focused at developing new DNA/RNA probe techniques and bioluminescent reporter system to measure the dynamics in microbial population density and activity during environmental biodegradation processes.

Metabolism of PAHs. *Pseudomonas fluorescens* 5RL contains a bioluminescent reporter plasmid (pUTK1) and exhibits a Nah^+Sal^- phenotype. In the ^{14}C-PAH mineralization study, strain 5RL can mineralizes [1-^{14}C]naphthalene (8.9 µCi/mmol; >98% purity) and [UL-^{14}C]anthracene (10.4 µCi/mmol; >98% purity) with the $^{14}CO_2$ recovery of 21.6% and 13.8%, respectively. The radiolabel in [9-^{14}C]phenanthrene (10.4 µCi/mmol; >99% purity) was not accessible, therefore no $^{14}CO_2$ was detected. *P. putida* 2440 (nah^-sal^-) and *P. fluorescens* 18H (nah^-sal^+) are used as negative controls.

Characterization of metabolites formed from anthracene and phenanthrene are carried out by biotransformation experiments. Incubation of the substrates without strain 5RL are used as abiotic control. Preliminary results from TLC indicated there are accumulation of metabolites in both studies. Identification of metabolites are further analyzed by HPLC and GC/MS. Two degradation products, 2-hydroxy-3-naphthoic acid and 1-hydroxy-2-naphthoic acid, are recovered and identified as biochemical metabolites from the biotransformation of anthracene and phenanthrene, respectively. This is the first study providing direct biochemical evidences of the naphthalene plasmid degradative enzyme system being involved in the degradation of higher molecular weight PAHs other than naphthalene.

Aerobic degradation of DDT (1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane). *Alcaligenes eutrophus* A5 is originally isolated by enrichment cultivation of polychlorinated biphenyl (PCB) contaminated sediments and is found to mediate a plasmid (pSS50) associated mineralization of 4-chlorobiphenyl through a 4-chlorobenzoic acid (4-CBA) intermediate. The strain A5 has lost the capacity to mineralize 4-CB over period of time, but retains a 4-CBA$^+$ phenotype. *A. eutrophus* A5 oxidizes DDT initially on the phenyl ring at the *ortho*- and *meta*- positions to produce hydroxylated-DDT intermediates with GC retention times at 19.55 and 19.8 min. Both intermediates share exactly the same mass spectra. A yellow ring-cleavage product, with maximum absorbance of 402 nm, has similar spectrophotometric characteristic as other *meta*-cleavage intermediates reported in the degradation of biphenyl and PCBs.

The result obtained from TLC radiochemical scanning indicating ^{14}C-DDT (291 μCi/mmol, 99.9% purity) is degraded to 4-CBA by comparison the R_f value with authentic standard. This intermediate, 4-CBA, is further confirmed by GC/MS analysis. The results of these current studies indicate

that strain A5 is capable of the aerobic degradation of DDT to 4-CBA via a *meta*-cleavage pathway.

Characterization of a substituted naphthalene catabolic pathway. *Pseudomonas* sp. strain JS1 has a broad substrate specificity on the oxidation of aromatic hydrocarbons, including naphthalene and salicylate. Preliminary results suggest that the naphthalene/salicylate pathway of Strain JS1 is different from the well-known pathway encoded by the NAH7 plasmid. Further demonstration by GC/MS show that both gentisate and salicylate are detected in the supernatant of naphthalene-grown culture. Also, gentisate is found in the salicylate-grown culture and gentisate dioxygenase activity is detected in salicylate-grown cell-free extract. Further enzyme assays showed that catechol 1,2-dioxygenase and catechol 2,3-dioxygenase activities are not detected in the cell-free extract of salicylate-grown culture. The above results strongly suggest that naphthalene is oxidized to salicylate, then further degrades via a gentisate pathway.

Several probes made form NAH7 genes, *nahA* (naphthalene dioxygenase gene), *nahG* (salicylate hydroxylase gene), *nahH* (catechol 2,3-dioxygenase gene) and *nahR* (positive regulatory protein), are probed with total DNA of JS1 under high stringency conditions. Only the *nahA* probe hybridized to the JS1 DNA which suggests the naphthalene dioxygenase of JS1 is related to that of NAH7 . The negative result of *nahR* probe indicates that either *nahR* gene is less conserved than *nahA* gene or the regulation of these two naphthalene pathways is controlled by two different genes

Construction of *lux* biodegradation reporter. A new *lux* bioluminescent reporter is developed to examine the co-oxidation of TCE by toluene dioxygenase in *P. putida* F1. A 2.75 kb DNA fragment containing the *tod* promoter is cloned in front of the promoterless *luxCABDE* genes on plasmid pUCD615. The resultant 20.3 kb plasmid, pUTK30, is triparentally mated from *E. coli* into *P. putida* F1. Under

toluene vapor, a light producing strain, B2, is selected for further study.

A differential volume reactor with the B2 cells encapsulated in alginate beads is used to examine the co-oxidation of TCE and its correlation to bioluminescence. This reactor system provides on-line monitoring on TCE degradation and bioluminescence response. The disappearance of TCE and toluene are on-line monitored by gas chromatography. Bioluminescence is monitored by a photomultiplier which is connected to a computer to record light output. The concentration of TCE is constant at 20 mg/l and toluene is pulsed into the reactor in square wave perturbations at 10 mg/l. Once toluene is added the bioluminescence (light output) increasing to a maximum level which maintains until toluene removed. Approximately 20% of TCE is degraded and 50% of toluene is removed at the flow rate of 0.4 ml/min. This result shows a correlation between the induction the *tod* operon monitoring by light output and the co-oxidation of TCE.

Screening new catabolic genotype using gene probe technique. The primary focus of this study is to discover higher PAH-degraders (3 or more rings). Environmental isolates (141) from bacterial culture collection are tested for determining their phenotypic characterizations. This is carried out using spray plate technique. Anthracene, phenanthrene, pyrene and benzo[a]pyrene are used as model PAHs. The organisms, that have the PAH-degrading capability, are further analyzed by indigo test, mineralization and colony hybridization.

Among 141 tested strains, 109 strains possess dioxygenase activity and 35, 81, 3 and 12 strains show clear zones on fluorene, phenanthrene, pyrene, and anthracene spray plates, respectively. Among naphthalene degraders, 4 strains did not hybridized with NAH7-derived gene probes (*nahA* and *nahG*) and their plasmids show different *Eco*RI and *Eco*RV restriction patterns from that of NAH7 plasmid. It is

possible that these 4 strains may have new *nah* genes which encode different naphthalene degradation pathway from conventional NAH7-encoded pathway. They may be a source to derive new gene probes. There are no isolates show clear zone on benzo[a]pyrene spray plates. However, a strain A8 shows ability to mineralize 7,10-^{14}C benzo[a]pyrene over 45% in 1/4 YEPG medium at 28°C for 7 days. A further investigation on the degradation of benzo[a]pyrene by strain A8 is undertaken.

Determination of bioluminescent response to PAH priority pollutants and metabolites. Two bioluminescent reporter strains *P. fluorescens* HK44 (*nah*$^+$, *sal*$^+$) and 5RL (*nah*$^+$, *sal*$^-$) are used in this study. 2 ml of exponentially growing culture (OD_{546}=0.35) are added to 2 ml of test solution in sterile 25 ml mineralization vials with teflon seals. PAH solutions are prepared in sterile water. Salicylate and naphthalene are used as positive control in the study. All experiments are conducted in triplicates.

The results of one-hour exposure assays with *P. fluorescens* HK44 using saturated solutions (without crystals) show that salicylate, naphthalene and acenaphthylene producing significant light increase as compared to the control without inducing substrates. All other compounds, acenaphthene, anthracene, benzanthracene, chrysene, dibenzanthracene, fluorene, fluoranthene, methyl-anthracene and phenanthrene, do not increase the catabolic *nahG* expression over the background control. In prolonged exposure assays (5.5 hrs) and the use of aqueous solutions containing crystals resulting in a significant induction of catabolic gene expression with several compounds which tested negatively in strain HK44, such as anthracene, benzo[a]pyrene, fluorene and phenanthrene. A similar bioluminescence response patterns are observed in the strain 5RL after 3.6 hour exposure to the PAHs. The above results suggest that the naphthalene degradation pathway in *P. fluorescens* HK44 and 5RL is not only induced after exposure

to naphthalene but might also be induced upon exposure to acenaphthene, acenaphthylene, fluorene, phenanthrene ,anthracene and benzo[a]pyrene.

In addition, it has been demonstrated with 5RL that the intermediate of anthracene degradation, 2-hydroxy-3-naphthoic acid, can act as an inducer of the naphthalene degradation pathway. But, the similar light induction response does not observed with 1-hydroxy-2-naphthoic acid, the intermediate in phenanthrene degradation.

Biosensing for pollutant availability for biodegradation in environmental systems. A bioassay is developed and standardized for rapid, quantitative and specific assessment of naphthalene and salicylate. The bioluminescent catabolic reporter strain *Pseudomonas fluorescens* HK44, which carries a transcriptional *nahG-luxCDABE* fusion for naphthalene and salicylate metabolism, is used. A good linear correlation between bioluminescence and naphthalene or salicylate aqueous phase concentration is found over a range of 1 to 2 orders of magnitude, using exponentially growing reporter cultures (OD_{546}=0.35). A significant bioluminescence response could be detected for naphthalene concentration of 45 ppb. Studies conduct under defined conditions with extracts and slurries of experimentally contaminated sterile soil and identical uncontaminated soil controls demonstrate that this method can be used for specific and quantitative estimations of target pollutant presence and bioavailability in soil extracts and for specific and qualitative estimations of naphthalene in soil slurries. In addition, the carbon starved resting cultures can cause significant bioluminescence increase upon exposure to readily metabolizable substrates such as glucose, peptone and yeast extract, thereby reducing the specificity of the response. Studies conduct in a ?ous extracts of environmental soil/sediment sample ontaminated with petroleum

hydrocarbons have demonstrated increasing bioluminescence response as compare to uncontaminated control samples.

The correlation between bioluminescence and biodegradation is examined in dual carbon substrate system, which containing 1 g/l glucose and 3 or 12 mg/l salicylate, using *Pseudomonas fluorescens* HK44 in a define mineral salts medium. Preliminary data show that a positive correlation exists between the integrated bioluminescence signal and the corresponding amounts of degraded salicylate.

Earlier studies demonstrated that bioluminescent reporter bacteria can be immobilized in calcium alginate which provides a translucent matrix. Exposure of *Pseudomonas fluorescens* HK44 immobilized in alginate brads to different naphthalene and salicylate concentrations result in transient bioluminescence responses. Good linear correlations are found between peak bioluminescence levels and target pollutant concentrations. Based on above results, the development of a continuous biosensor for monitoring pollutant bioavailability in waste streams is under taken.

Publications associated with this investigation.

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Layton, A.C., J. Sanseverino, W. Wallace, C. Corcoran and G.S. Sayler (1992). Evidence for 4-chlorobenzoic acid dehalogenation mediated by pSS50 related plasmids. Appl. Environ. Microbiol. 58:3012-3019.

Menn, F.-M., B.M. Applegate and G.S. Sayler. NAH plasmid mediated catabolism of anthracene and phenanthrene to naphthoic acids. (manuscript submitted)

Nadeau, L., F.-M. Menn, A. Breen and G.S. Sayler. Degradation of DDT by *Alcaligenes eutrophus*. (manuscript in preparation)

Sanseverino, J., B.M. Applegate, J.M. Henry King and G.S. Sayler (1992). Plasmid-mediated mineralization of naphthalene, phenanthrene and anthracene. (manuscript submitted)

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Wallace, W. and G.S. Sayler (1992). Plasmids in the environment. In: Encyclopedia of Microbiology, Vol.1. J. Leberberg (ed), pp.417-430, Academic Press.

Presentations and Abstracts

Applegate, B., J. McPherson, F.-M. Menn, A. Heitzer and G.S. Sayler (1992). Application of bioluminescent reporter technology as a tool to investigate the involvement of the Nah system in the catabolism of different polyaromatic hydrocarbons. Abstract Q-64, p.346. American Society for Microbiology (ASM) Annual Meeting, New Orleans, LA.

Callicotte, L., J. Sanseverino, B. Applegate and F.-M. Menn (1992). Preliminary characterization of the naphthalene catabolic pathway of *Pseudomonas* sp. JS1. Abstract Q-66, p.346. American Society for Microbiology (ASM) Annual Meeting, New Orleans, LA.

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Huang, B., T.W. Wang, R. Burlage and G.S. Sayler (1992). Development of an on-line sensor for bioreactor operation. Proceeding of the Fourteenth Symposium on Biotechnology for Fuels and Chemicals.

Johnston, W. and G.S. Sayler (1992). Maintenance and stability of nah-lux bioluminescent reporter strains and plasmids. American Society for Microbiology (ASM) Annual Meeting, New Orleans, LA.

Sayler, G.S. (1992). Environmental biotechnology's future in hazardous waste management. Third Pacific Rim Biotechnology Conference, Taipei, Taiwan.

Sayler, G.S. (1992). Bioluminescent monitoring of degradative gene expression in soils. American Society of Agronomy. Minneapolis, Minnesota.

Sayler, G.S. (1992). Molecular approaches for diagnostics, performance monitoring and optimization in bioremediation. U.S./Israel Bioremediation Workshop, Tel Aviv, Israel.

Lectures and Seminars (G.S. Sayler, 1992)

Virginia Tech and State University, Department of Biology, Blacksburg, VA. Invited seminar.

Sigma XI - Southern Appalachia Regional Lecture, ETSU, Johnson City, TN. Invited lecture.

University of North Carolina Charlotte, Department of Biology, Environmental Biotechnology Lecture. Invited lecture.

Rensselaer Polytechnic Institute, Department of Biology, Troy, NY. Invited Bray lecture.

EPA Cincinnati, Environmental Research Laboratory, Biotechnology Lecture, Invited lecture.

Third Pacific Rim Biotechnology Conference, Taipei, Taiwan. Invited lecture.

American Society of Agronomy. Minneapolis, Minnesota. Invited lecture.

U.S./Israel Bioremediation Workshop, Tel Aviv, Israel. Invited lecture.