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14. ABSTRACT

Biomarkers for monitoring *in situ* biodegradation of PAHs in anoxic sediments were developed by careful study of microbial degradation mechanisms. Both metabolic and molecular (genetic) biomarkers were studied. Several unique metabolites of anaerobic naphthalene degradation were identified and methods were developed for detecting them in environmental samples. Detection of these metabolic biomarkers at several contaminated sites has demonstrated their usefulness. The other biomarker that was developed was the *dsrAB* gene. A comparison of many different sequences of this gene allowed us to identify sequence groups that are correlated to PAH and petroleum hydrocarbon degradation.

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Biomarkers, PAH, Anaerobic, Biodegradation, Sediment

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Final Report

Grant #: N00014-99-0083

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Institution: Biotechnology Center for Agriculture and the Environment
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Co-Principle Investigator: Lee J. Kerkhof, Ph.D.

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Grant Title: Biomarkers for Monitoring *In-situ* Biodegradation of PAHs in Anoxic Harbor Sediment

Award Period: 1 October 1998 - 30 September 2002

Objective: The long-term objective of this project was to develop biomolecular, chemical and genetic tools to detect *in-situ* biodegradation of aliphatic and aromatic petroleum constituents in anoxic sediments. The specific objectives were a) to characterize and to validate metabolic biomarkers that are specific to the anaerobic biodegradation of PAH components; and b) to develop a PCR based assay for determining *in-situ* activity using the dissimilatory sulfite reductase target genes implicated in bioremediation of impacted sediment.

Approach:

GC/MS analyses of unlabeled and stable isotope-labeled substrates in active consortia were used to identify unique metabolites and provide insight into the anaerobic pathways of BTX, alkane and PAH degradation. The naphthalene metabolites that we identified were used as bioindicators of anaerobic microbial activity. These included 2-NA (2-naphthoic acid), TH-2-NA (tetrahydro-2-naphthoic acid), HH-2-NA (hexahydro-2-naphthoic acid) and MNA (methylnaphthoic acid), all of which are unusual metabolites and specific to the anaerobic biodegradation pathway observed in laboratory studies. GC/MS was used to examine two contaminated groundwater sites for PAH biomarkers as evidence of anaerobic natural attenuation. One of the sites is an underground storage tank leak on the East Coast and the second is a creosote wood treatment facility on the West Coast. In addition to these sites, sediment samples from Norfolk Harbor were analyzed for the same suite of metabolic bioindicators. These samples were obtained on the January and June 2002, cruises conducted by the US Naval Research Lab.

The nucleic acid based part of the project has focused on identifying microbial dissimilarity sulfite reductase (*dsrAB)* genes [exclusive to SRB's]that are transcribed in natural samples and are correlated to anaerobic aromatic degradation. This approach is rapidly gaining acceptance, with over 450 *dsrAB* entries currently in Genbank. In order to characterize large numbers of environmental samples, we have used a rapid fingerprinting technique utilizing fluorescent end labeling of PCR product (target genes) and screening by terminal restriction length polymorphism (TRFLP). Samples from a wide variety of contaminated and uncontaminated sediments from around the world were

collected. The *dsrAB* genes in these sediments were PCR amplified, identified and examined for relatedness and similarity.

Accomplishments:

1) Stable isotope-labeled substrate studies in active consortia were used to identify unique metabolites of the anaerobic pathways of BTX, alkane and PAH degradation. These studies resulted in publication of proposed degradation mechanisms for naphthalene, methylnaphthalene and phenanthrene (Zhang, et al., 2001; Sullivan, et al., 2001) benzene (Phelps, et al., 2001) and medium chain-length alkanes (So and Young, 1999; So, et al., 2003) in sulfate-reducing sediments.

2) Extraction and analytical methods were developed to detect unique metabolites of anaerobic PAH degradation from groundwater and sediment samples. These methods were used to show that these metabolites could be used as biomarkers to identify areas of *in situ* biodegradation in a fuel-contaminated aquifer (Phelps, et al., 2002).

3) Analysis of sediment samples from a series of well-studied stations in Norfolk Harbor, VA showed that one of the biomarkers (2-naphthoic acid) was detectable at some, but not all, of the stations. In addition, other carboxylic acids that may be useful for diagnosing anaerobic PAH biodegradation were found to be present at some sites.

4) PCR primers were designed and used successfully to amplify new *dsrAB* genes from sulfate-reducing BTEX, alkane and PAH degrading consortia. These new sequences were used to significantly refine our understanding of sulfate-reducer phylogeny and to identify sequence groups that are associated with PAH biodegrading communities (Perez-Jimenez, et al., 2001).

5) Optimization of restriction enzymes for TRFLP analysis has been completed and "fingerprints" of the *dsrAB* genes from polluted and non-polluted sites in the Eastern and Western USA, Latvia, Korea, Puerto Rico, Venezuela, and Italy have been obtained. Comparison of these gene patterns shows that there are differences that can be interpreted as biomarkers of biodegradation.

Conclusions: ONR support of our project has resulted in a knowledge base about the anaerobic fate of PAHs and alkanes that had not existed before. Our work has demonstrated that: 1) PAHs and alkanes can be fully biodegraded by anaerobic microorganisms found in impacted harbor sediment; 2) the mechanisms of PAH degradation in the absence of oxygen include carboxylation via inorganic bicarbonate; 3) a sequential reduction of the unsubstituted ring takes place prior to ring fission; 4) metabolites of anaerobic PAH degradation found in groundwater contaminated sites include 2-naphthoic acid, tetrahydro-2-naphthoic acid, hexahydro-2-naphthoic acid and methyl-2-naphthoic acid; 5)Norfolk Harbor sediment contain some of these same metabolites as well as other carboxylic acids that may result from biodegradation; 6) over 450 different *dsrA* TRF's can be detected in sediment samples from different geographical areas around the world with around 25% being represented in Genbank or clonal libraries. These results suggest a large number of *dsrA* genes remain unknown. Furthermore, these novel biochemical reactions may be used for monitoring natural attenuation and underscore the metabolic diversity which is found in the natural microbial population.

Significance: The new understanding of anaerobic hydrocarbon degradation that has come from this project will be very useful for helping policy makers and site managers monitor populations and activity in impacted sediments in order to optimize management and remediation efforts.

Patent Information: No patents have been applied for.

Award Information:

2002. L.Y. Young was awarded the American Society for Microbiology, National Proctor and Gamble Award in Applied and Environmental Microbiology.

Publications and Abstracts:

Peer Reviewed Publications:

1. So CM & LY Young. 1999. Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. Appl. Environ. Microbiol. 65:2969-2976.

2. So CM & LY Young. 1999. Initial reactions in anaerobic alkane degradation by a sulfate-reducer, strain AK01. Appl. Environ. Microbiol. 65:5532-5540.

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5. Phelps, CD, X Zhang and LY Young. 2001. Use of stable isotopes to identify benzoate as a metabolite of benzene degradation in a sulfidogenic consortium. Env. Microbiol. 3(9):600-603.

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7. Sullivan ER, X. Zhang, C Phelps & LY Young. 2001. Anaerobic mineralization of stable isotope labeled 2-methylnaphthalene. Appl Environ Microbiol 67(9):4353-4357.

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9. Phelps CD & LY Young. 2001. Biodegradation of BTEX under Anaerobic Conditions: a Review. Adv. In Agronomy, vol 70, pp. 329-357.

10. Phelps CD, J Battistelli & LY Young. 2002. Metabolic biomarkers for monitoring anaerobic naphthalene biodegradation *in situ*. Environ Microbiol 4:532-537.

11. So CM, CD Phelps & LY Young. 2003. Anaerobic transformation of alkanes to fatty acids by the sulfate-reducing bacterium, strain Hxd3. Appl. Environ. Microbiol. (in press).

Poster Presentations and Abstracts:

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2. Liang W, KJ Rockne, L Shor, DS Kosson, GL Taghon & LY Young. 2000. Bioavailability of field-aged polycyclic aromatic hydrocarbons on Piles Creek, NJ and New town Creek NY sediment. Presentation and Abstract for the Ann Mtg Am Soc Microbiol.

3. Perez J, E Sullivan, LY Young & L Kerkhof. 2000. Molecular characterization of sulfate-reducing bacteria in anaerobic AH degrading consortia using dissimilitory sulfite reductase (*dsrA*) genes. Presentation and Abstract for the Ann Mtg Am Soc Microbiol.

4. Phelps CD, J Oberer, G Mortimer & LY Young. 2000. Use of metabolic biomarkers to monitor anaerobic *in-situ* bioremediation at a contaminated field site. Presentation and Abstract for the Ann Mtg Am Soc Microbiol.

5. Scala D, E Hacherl, R Cowan, LY Young & DS Kosson. 2000. Molecular characterization of Fe(III) reducing enrichment cultures using T-RFLP analysis. Presentation and Abstract for the Ann Mtg Am Soc Microbiol.

6. Liang W, K Rockne, LM Shor, DS Kosson, GL Taghon & LY Young. 2001. The effect of aging on the bioavailability of phenanthrene in both low- and high-organic matter sediment. Presentation and Abstract for the Ann Mtg, Amer Soc Microbiol.

7. Sullivan E, X Zhang, LY Young. 2001. Mineralization of 2-methylnaphthalene through 2-naphthoic acid by a sulfate reducing consortium. Presentation and Abstract for the Ann Mtg, Amer Soc Microbiol.

8. Phelps C, LY Young, X Zhang. 2001. Transformation of biphenyl and fluorobiphenyl by a sulfidogenic consortium degrading phenanthrene. Presentation and Abstract for the Ann Mtg, Amer Soc Microbiol.

9. J. Perez-Jimenez, L. Young, and L. Kerkhof. 2001. Genetic Diversity of Sulfate Reducing Bacteria in a Contaminated and a Pristine Environment. Presentation and Abstract for the Ann Mtg, Amer Soc Microbiol.

10. J. R. Pérez-Jiménez, L. Y. Young, L. J. Kerkhof 2002. Sulfidogenic Communities in Tropical Mangrove Forest Soils Revealed by the Dissimilatory Sulfite Reductase (*dsrAB*) Genes. Presentation and Abstract for the Ann Mtg, Amer Soc Microbiol.

11. Battistelli J, CD Phelps & LY Young. 2002. Use of metabolic biomarkers for monitoring anaerobic naphthalene biodegradation. Presentation and Abstract for the Ann Mtg, Amer Soc Microbiol.

Platform Presentations:

1. Phelps CD & LY Young. 2000. Anaerobic metabolites and co-metabolites as bioindicators of *in situ* biodegradation. Annual Conference of Northeast Microbiologists for Physiology, Ecology, Taxonomy. Blue Mtn. Lake, NY.

2. Phelps CD & LY Young. 2000. Metabolic biomarkers as indicators for anaerobic *in situ* remediation of BTEX and PAH. Remediation Technologies Development Forum, Wilmington, DE, Sept. 12-13, 2000.

3. Phelps CD & LY Young. 2001. Metabolic biomarkers for monitoring *in situ* toluene and naphthalene bioremediation. Battelle Symposium on In Situ and On-Site Bioremediation, San Diego, CA.

4. Young LY. 2002, "Anaerobic Processes in the Environment and the Biodegradation of Hydrocarbons and Related Compounds", awards lecture. American Society for Microbiology, National Proctor and Gamble Award in Applied and Environmental Microbiology. Annual Meeting, Salt Lake City, UT.

5. Young LY. 2002. Anaerobic metabolism of naphthalene and alkanes. ASM Symposium on Recent Developments in anaerobic Hydrocarbon Degradation, American Society for Microbiology Annual Meeting, Salt Lake City, UT.

6. Young LY. 2002. Microbial biomarkers for monitoring in-situ anaerobic hydrocarbon degradation. Bioremediation and Biodegradation: Current advances in reducing toxicity, exposure and environmental consequences, June 2002, Asilomar, CA.

7. Phelps CD & LY Young. 2002. Metabolic biomarkers for detecting anaerobic PAH biodegradation in groundwater and sediments. 18th Annual International Conference on Cotnaminated Soils, Sediments and Water, Oct. 21-24, 2002, Amherst, MA.

8. José R. Pérez-Jiménez, Lily Y. Young, & Lee J. Kerkhof. Sulfidogenic Communities in Neotropical Mangrove Forest Soils Revealed by the Dissimilatory Sulfite Reductase (*dsrAB*) Genes. Annual Biomedical Research Conference for Minority Students. November 13-16, 2002 New Orleans, Louisiana