

# **Environmental Management Science Program**

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## **Rapid Mass Spectrometric DNA Diagnostics for Assessing Microbial Community Activity During Bioremediation**

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### Research Objective

Biological activity has often been attributed to changes in pollutant profiles found in contaminated soils when abiotic processes actually caused pollutant removal. Careful evaluation of bioremediation necessitates that all transformation and removal pathways are anticipated so that the pathways are controlled or monitored. Our work is directed at evaluating a monitoring strategy that relies on the combined use of DNA diagnostic procedures and mass spectrometry as the detection scheme. The intent is to track bioremediation by measuring the occurrence of genes in soil samples that are known to code for enzymes capable of degrading specific pollutants. Matrix-assisted-laser-desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) offers the possibility for automation and high throughput PNA detection as is needed to track the course of bioremediation over large polluted areas.

### Research Progress and Implications

As of last year, the first year in a three year project, we identified and sequenced a unique 161-bp region useful for identifying the nahH gene. In this second year of the project, two approaches were used to probe DNA samples for the presence of this naphthalene degrading gene. The first was a Ligase Chain Reaction (LCR). In a reaction where the DNA target template is present, LCR reaction conditions were established to detect a 40-bp section of the gene by ligating two 20-bp oligonucleotide probes designed to anneal abuttingly to this target. The detection of a 40-mer oligonucleotide serves to score the test positive. In the absence of the specific target sequence, ligation should not occur and the test is scored negative. The products of the ligation reactions were analyzed by an assay incorporating alkaline phosphatase onto one of the oligonucleotides. The LCR approach is very sensitive, correctly identifying the presence of nahH using sub-attomole quantities of template.

The second approach we examined involved the use of Peptide Nucleic Acid (PNA) probes to identify a Polymerase Chain Reaction (PCR) amplicon from nahH. In this assay, double-stranded PCR products are affinity-captured to streptavidin-coated magnetic beads by means of a biotin label on one of the PCR primers. The non-biotinylated strand of DNA is denatured and removed in a wash step, after which a PNA oligomer is allowed to anneal to the captured strand. Excess PNA is then washed away while affinity-captured PNA is transferred to a MALDI-TOF-MS sample holder. The PNA is released from the complex when matrix is added. The test is scored positive when a peak corresponding to the mass of PNA<sup>+</sup> appears in the mass spectrum.

We observed that the PNA assay offers several advantages over the LCR approach and other existing analytical techniques for identifying the presence of genes in environmental DNA samples. PCR reactions are much less susceptible to changes in reagent concentrations than are LCR reactions, leading to higher confidence in procedures based on PCR. PNA-DNA complexes tend to be more stable than corresponding DNA-DNA complexes and therefore hybridization is easier to control. Single-base mismatches disrupt PNA-DNA stability far more than they do DNA-DNA stability, which increases the diagnostic value of the procedure.

We investigated the use of a PNA probe and MALDI-TOF-MS as a way to detect the naphthalene-degrading di-oxygenase gene in environmental soil samples. We then studied the reliability of this test by varying reaction conditions. DNA extracted from a bacterial colony known to contain naphthalene-degrading genes was amplified by PCR and allowed to hybridize with a 14-mer PNA probe. The bead-DNA-PNA complex was washed several times and added to a sinapinic acid (SA) solution. SA is used in this procedure as the MALDI matrix. The mixture was transferred to a

MALDI probe and evaporated under nitrogen. 100 fmol of pure 14-mer PNA is the lower practical limit of detection for PNA in SA measured with our MALDI-TOF mass spectrometer. The intensity of the PNA<sup>+</sup> signal generated with standards increased linearly with [PNA].

When bead-DNA-PNA complex was mixed with SA, PNA was not released efficiently from the target DNA unless 0.1% TFA was present. In the presence of TFA, the resulting bead-DNA, PNA and matrix solution could be evaporated to form a crystallized MALDI sample. Examination of the crystals under the microscope revealed many of the beads were incorporated into the crystals and sometimes interfered with the formation of crystals. The presence of the beads reduced the limit of detection to about 500 fmole of PNA. A plot of PNA<sup>+</sup> signal intensity vs. the concentration of PCR product, for a fixed concentration of the PNA probe, rises linearly. We also found that it was important to carefully optimize the conditions for denaturing the double-stranded PCR product after it was affinity captured. Even though it has been reported that Na<sup>+</sup> attaches less tenaciously to PNA than DNA, we frequently observed PNA mass spectra with 1 to 4 sodium ions attached to a 14-mer PNA. For this reason we attempted to denature the PCR product with 100% NH<sub>4</sub>OH but found it to be inefficient.

### **Planned Activities**

The MALDI technique we developed could be used to analyze a large number of samples rapidly. We are evaluating MALDI hardware modifications for conducting the analyses under computer control, thus providing a high throughput capability needed to track the course of bioremediation. The technique is easily adaptable for use in many different types of studies because it is possible to redesign the PCR primers and the PNA probe for the purpose of detecting a large number of different genes as needed to monitor the degradation of several pollutants. We will attempt to measure the occurrence of the di-oxygenase gene in environmental samples contaminated with naphthalene.