

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

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EXECUTIVE SUMMARY

The effort of the past year's activities, which covers the first year of the project, was directed at developing DNA-based diagnostic procedures for implementation in high through-put analytical instrumentation. The diagnostic procedures under evaluation are designed to identify specific genes in soil microorganisms that code for pollutant-degrading enzymes. Current DNA-based diagnostic procedures, such as the ligase chain reaction (LCR) and the polymerase chain reaction (PCR), rely on gel electrophoresis as a way to score a diagnostic test. We are attempting to implement time-of-flight (TOF) mass spectrometry as a replacement for gel separations because of its speed advantage and potential for sample automation. We anticipate that if TOF techniques can be implemented in the procedures, then a very large number of microorganisms and soil samples can be screened for the presence of specific pollutant-degrading genes.

1.0 OBJECTIVE

The use of DNA-based procedures for the detection of biodegrading organisms or genes that code for pollutant-degrading enzymes constitutes a critical technology for following biochemical transformation and substantiating the impact of bioremediation. DNA-based technology has been demonstrated to be a sensitive technique for tracking micro-organism activity at the molecular level. These procedures can be tuned to identify groups of organisms, specific organisms, and activity at the molecular level. We are developing a monitoring strategy that relies on the combined use of DNA diagnostics with mass spectrometry as the detection scheme. The intent of this work is a two-fold evaluation of 1) the feasibility of replacing the use of gel separations for identifying polymerase chain reaction (PCR) products with a rapid and automatable form of electrospray mass spectrometry and 2) the use of matrix-assisted-laser-desorption-ionization mass spectrometry (MALDI-MS) as a tool to score oligonucleotide ligation assays (OLA).

2.0 APPROACH

We have chosen to investigate TOF procedures that detect 1) when two approximately 20-base long oligonucleotides are ligated, 2) PCR products in the 100 bp size range, 3) PCR products in the megadalton size range, i.e., ≥ 1500 bp and 4) plasmids which carry catabolic genes. This course of investigation is based on performance characteristics of current mass spectrometry capabilities under development in our laboratory. We began by developing the necessary molecular biology procedures and then started to evaluate the use of matrix-assisted-laser-desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and charge detection mass spectrometry (CDMS) as detection schemes^{1,2}.

The naphthalene enzymatic-degradation pathway is shown in the following schematic³. In figure 1, capital letters refer to the various steps in the pathway. We have focused on steps C and H which occur as a result of the activity of the nahC (1,2-dihydroxynaphthalene oxygenase) and nahH (catechol oxygenase) enzymes, respectively.

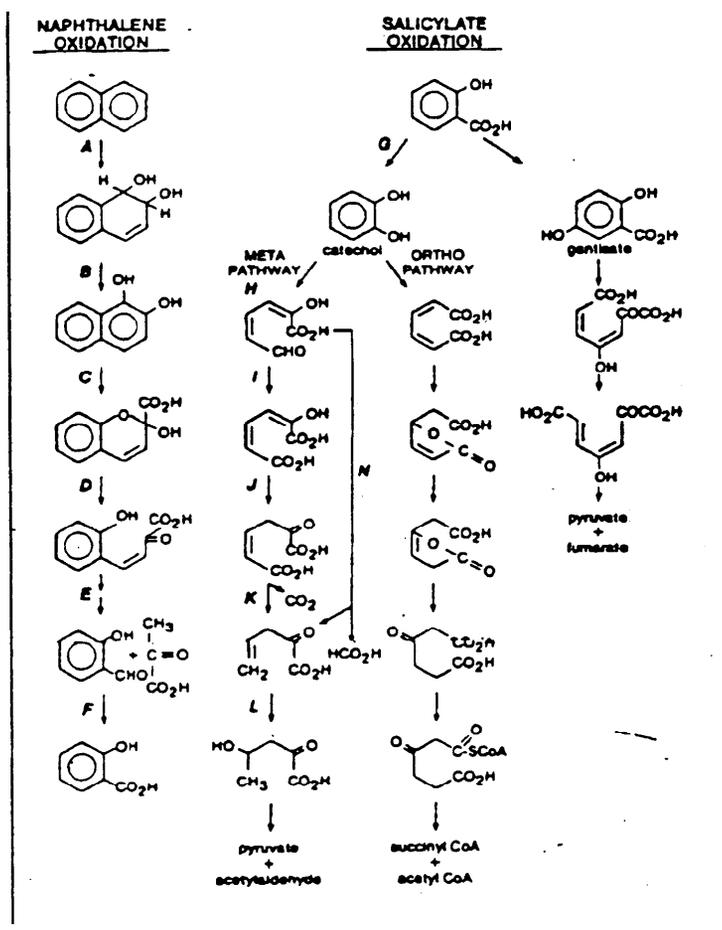


Figure 1. Naphthalene degradation pathway

Pseudomonas .stutzeri P-16, an organism observed by W. Stringfellow, Center for Environmental Biotechnology, LBNL, to grow using naphthalene as its only carbon source was selected as a source of DNA for testing our proposed diagnostic procedure. A GenBank search for genes coding for nahH and nahC revealed that several highly similar sequences are shared by a few strains of *Pseudomonas*. Regions of high sequence similarity in nahH and nahC were used to direct the design of primers for the LCR. Genomic DNA was isolated from *Pseudomonas stutzeri* P-16 and used as a target in an LCR assay. We were unable to obtain a positive LCR test even when 1008 degenerate primers were utilized in the assay to compensate for several uncertainties in the target sequence. The negative results were assumed to be caused by the sequence uncertainties and the resulting low concentrations of degenerate primers introduced into the reaction. Therefore, we attempted to sequence a short section of the nahH gene to confirm or disprove its presence in P-16, and if present, use the sequence to design unique primers. Primers were selected to amplify a 161-bp region and PCR generated the expected product. We observed that the sequence we determined is very similar, yet unique, to genes for catechol oxygenase.

Using the sequence obtained for the 161-bp region of the nahH gene, a new set of LCR oligonucleotides was selected and a series of reactions was run to optimize product amplification while simultaneously minimizing template-independent ligation. A colorimetric assay using an alkaline phosphatase labeled primer was used to analyze the results of the reactions. The following parameters were investigated: primer concentration, template concentration, non-homologous (salmon sperm) DNA concentration, ligase concentration, and thermal cycle number. The following parameters yielded the best ratio of positive amplification to negative control for a 50 μ L reaction:

- Primer Concentration: 10 fmol/ μ L
- Template Concentration: 20 zmol/ μ L
- Salmon Sperm Amount: 250 ng
- Ligase Amount: 2 units
- Thermal Cycle Number: 32

With this set of primers and primer binding conditions we were able to detect the nahH gene in genomic DNA isolated from P-16 using a LCR diagnostic procedure. This establishes the completion of one milestone of the project, namely the demonstration of a test useful for detecting a specific gene in a pollutant-degrading organism. We are currently working to establish an appropriate magnetic bead-based affinity capture technique for use in concentrating and desalting the ligated product so that it can be identified by MALDI-TOF-MS.

In an attempt to evaluate the possible application of CDMS as a tool for detecting large PCR products, we generated a 1982 bp long strand using pUC19 as template DNA. This template was chosen because the conditions and primers were readily available and thus

provided a quick way to test the capability of CDMS in this particular application. In the CDMS technique, electrospray converts DNA molecules in solution into highly charged gas-borne DNA ions. The ions are then directed to fly through a Faraday tube where their image charge signal is measured. This leads to a mass measurement of individual ions. When applied to PCR products in the megadalton size range, a mass measurement provides a way to determine the size of the DNA strand. Mass measurements can be acquired from as few as about 1000 ions in a time period as short as about 10 min. When this procedure is compared to the several hours of time needed to separate the PCR product on an electrophoresis gel, the time saving is obvious. Figure 2 shows a mass spectrum of the 1982 bp PCR product determined with the CDMS technique.

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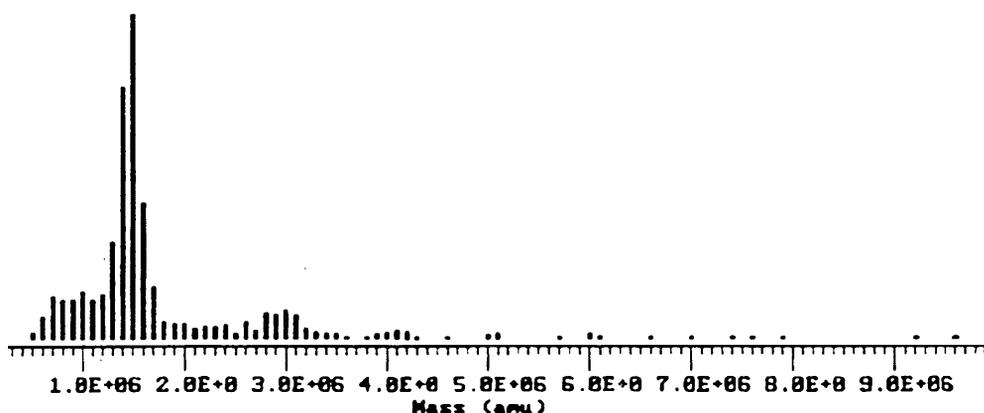


Figure 2. Mass spectrum of a 1982 bp (1.3 MDa) PCR product.

The assay that we are developing is specific to naphthalene and can be used to track the bioremediation of naphthalene. Future work will be directed towards transforming this technique into a more general procedure so that it could be applied to the investigation of other pollutants and their degradation pathways.

¹Fuerstenau, S.D. and W.H. Benner, Molecular Weight Determination of Megadalton DNA Electrospray Ions Using Charge Detection Time-of-Flight Mass Spectrometry, Rapid Commun. Mass Spectrom., 9, 1528-1538, 1995.

²Schultz, J.C., C. A. Hack and W.H. Benner, Mass Determination of Megadalton-DNA Electrospray Ions using Charge Detection Mass Spectrometry, submitted to J. Am. Soc. Mass. Spec., 1997.

³Yen, K.-M. and C. M. Serdar, Genetics of Naphthalene Catabolism in Pseudomonads, CRC Critical Rev. in Microbial., 15, 247-263, 1988.