

FINAL REPORT
U.S. Department of Energy

**Monitoring Genetic and Metabolic Potential for *in situ* Bioremediation:
Mass Spectrometry**

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Executive Summary

A number of DOE sites are contaminated with mixtures of dense non-aqueous phase liquids (DNAPLs) such as carbon tetrachloride, chloroform, perchloroethylene, and trichloroethylene. At many of these sites, *in situ* microbial bioremediation is an attractive strategy for cleanup, since it has the potential to degrade DNAPLs *in situ* without producing toxic byproducts or expensive removal procedures. A rapid screening method to determine broad range potential for contaminant degradation by microbes would greatly reduce the cost and time involved in assessment for *in situ* bioremediation, as well as for monitoring ongoing bioremediation treatment.

The objective of this project was the development of mass-spectrometry-based methods to screen for genetic potential for both assessment and monitoring of *in situ* bioremediation of DNAPLs. These methods were designed to provide more robust and routine methods for DNA-based characterization of the genetic potential of subsurface microbes for degrading pollutants. Specifically, we sought to 1. Develop gene probes that yield information equivalent to conventional probes, but are more amenable to mass spectrometric detection, 2. Pursue improvements to mass spectrometry methodology in order to allow more general application to gene probe detection, 3. Increase the throughput of microbial characterization by integrating gene probe preparation, purification, and MALDI-MS analysis.

In this EMSP project (for which funding ended in March 2000), we demonstrated proof of principle for the idea of using mass spectrometry as a readout technique for gene probe assays of microbes relevant to bioremediation. The project was a collaborative effort between Oak Ridge National Laboratory (ORNL) and Professor Mary Lidstrom's group at the University of Washington.

Prof. Lidstrom's group developed molecular techniques for analyzing natural populations of methanotrophic bacteria. A DNA sequence database was developed for methanotrophs in Lake Washington sediment, and used to design assays specific for selected diagnostic genes in methanotrophs. These molecular tools were used to analyze the natural populations of methanotrophs in Lake Washington sediments by conventional hybridization techniques. A major surprise from this work was the finding that a significant segment of the natural population are sMMO-containing *Methylomonas* strains, which are of interest because they have the potential to carry out high rate degradation of trichloroethylene. New primers have been designed to detect this group of organisms.

At ORNL, we have used these tailored products as a model system in developing a streamlined MALDI-MS protocol. We have characterized these products and examined the effects of interferences on our protocol. Aspects of this protocol include a rapid method for preparing PCR products for MALDI-MS analysis, parallel implementation of this purification, and automated MALDI-MS data acquisition. We have achieved advances in figures of merit for MALDI-MS analysis of PCR products, including mass range, resolution, and reproducibility.

Relevance, Impact, and Technology Transfer: The issues addressed by this project involve technology to increase the applicability of *in-situ* bioremediation for DNAPL, or potentially any microbially-degraded pollutant. We have applied fundamental knowledge in molecular biology and analytical chemistry to the problem of rapidly characterizing microbial populations at potential cleanup sites. The project was a collaboration between the Organic and Biological Mass Spectrometry group at Oak Ridge National Laboratory (ORNL), and Professor Mary Lidstrom's research group at the University of Washington. Two Ph.D. students in Prof. Lidstrom's laboratory worked on this project; Andria Costello's thesis, "Molecular Ecology

Studies of Methanotrophs in a Freshwater Lake Sediment,” (available as UMI # 9930380 from UMI, 300 N. Zeeb Rd, Ann Arbor, MI) describes their results, and Ann Auman is currently completing her thesis research. Two postgraduate research associates, Dr. Yongseong Kim and Ms. Kristal Weaver, participated in the research at ORNL. While the work resulted in proof of principle for mass spectrometry-based detection of microbial gene probes, further work needs to be done to develop a wider array of suitable probes, and to refine the mass spectrometry methodology before the knowledge gained in the project will be directly applicable to DOE Environmental Management problems.

Research Objectives

A number of DOE sites are contaminated with mixtures of dense non-aqueous phase liquids (DNAPLs) such as carbon tetrachloride, chloroform, perchloroethylene, and trichloroethylene. At many of these sites, *in situ* microbial bioremediation is an attractive strategy for cleanup, since it has the potential to degrade DNAPLs *in situ* without the need for pump-and-treat or soil removal procedures, and without producing toxic byproducts. A rapid screening method to determine broad range metabolic and genetic potential for contaminant degradation would greatly reduce the cost and time involved in assessment for *in situ* bioremediation, as well as for monitoring ongoing bioremediation treatment.

The objective of this project was the development of mass-spectrometry-based methods to screen for genetic potential for both assessment and monitoring of *in situ* bioremediation of DNAPLs. These methods were designed to provide more robust and routine methods for DNA-based characterization of the genetic potential of subsurface microbes for degrading pollutants. Specifically, we sought to 1. Develop gene probes that yield information equivalent to conventional probes, but in a smaller size that is more amenable to mass spectrometric detection, 2. Pursue improvements to matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) methodology in order to allow its more general application to gene probe detection, 3. Increase the throughput of microbial characterization by integrating gene probe preparation, purification, and MALDI-MS analysis.

Effective decision-making regarding remediation strategies requires information on the contaminants present and the relevant hydrogeology. However, it also should include information on the relevant bacterial populations present and the biodegradative processes they carry out. For each site at which bioremediation is considered, it is necessary to determine whether sufficient intrinsic degradative capability is present to suggest intrinsic bioremediation as a viable option, or whether a strategy involving addition of specific nutrients is more likely to be successful. In addition, if the existing genetic potential does not include the desired processes, it may be necessary to add external organisms as well as nutrients, which would negatively impact cost and feasibility scenarios. Once a bioremediation strategy is decided upon and initiated, it is important to carry out monitoring of the bacteria and their activities. Real-time data of this type during the treatment process can allow ongoing evaluation to optimize biodegradation, reducing cost and avoiding possible toxic byproducts. Clearly, the development of novel bioremediation technologies and informed decision-making regarding bioremediation as a treatment option will require in-depth information on the bacteria present at each site and the processes they carry out. Currently such information is generated by labor- and time-intensive treatability tests in the laboratory, and these do not generally assess a broad range of metabolic processes. We undertook this project because a rapid screening method to evaluate genetic potential is an important development to reduce costs for implementing *in situ* bioremediation strategies at DOE sites.

At the outset of this project, it was clear that the explosion of information in the DNA sequence database raised the possibility of developing diagnostic DNA signatures for key microbial processes, as a means for assessing genetic potential. The methods developed in our project would be able to take advantage of the growing information on sequences from environmental samples as well as from microbial genome sequencing projects. An increasing number of metabolic functions could be screened as the depth of information available for designing diagnostic sequences increased.

To apply the growing microbial sequence data to characterization of microbial populations, it is necessary to be able to detect specific DNA signature sequences. The application of gene probes to characterization of metabolic potential of microbial populations at the DOE Savannah River Site has been carried out using methods similar to these [Bowman *et al.*, 1993]. Current methods involve amplifying DNA using the polymerase chain reaction (PCR) or other amplification procedures to make multiple copies of a characteristic DNA fragment [Gibbs, 1990]. Detection of PCR products has conventionally been performed by gel electrophoresis or by hybridization with a complementary, labeled DNA probe. These techniques can be slow or yield ambiguous results, do not lend themselves to rapid, routine screening of many samples, and are generally viewed as the major bottleneck in many molecular biology laboratories.

To eliminate this bottleneck, we set out to perform research that would allow the use of mass spectrometry as a replacement “readout” technology for gene probe assays. Mass spectrometry is a method for measuring mass-to-charge ratios (m/z) of gas-phase ions. In the 1980’s, advances in ionization processes, such as matrix-assisted laser desorption/ionization (MALDI) [Hillenkamp *et al.*, 1991] and electrospray (ES) ionization [Fenn *et al.*, 1989] allowed analysis of large biomolecules, including DNA oligomers, by mass spectrometry. When used for detection of PCR products, mass spectrometry yields information similar to electrophoresis, with DNA size information read directly from the peaks in the mass spectrum, but with analysis times reduced by several orders of magnitude compared to electrophoresis [Doktycz *et al.*, 1995; Bai *et al.*, 1995; Wunschel *et al.*, 1996]. The Organic and Biological Mass Spectrometry Group at ORNL has developed a mass spectrometry-based method for identifying small DNA signature molecules produced by PCR amplification from natural samples. [Doktycz *et al.*, 1995] The efficacy of this concept was demonstrated by successful amplification and MALDI-MS detection of PCR products from *Legionella pneumophila* [Hurst *et al.*, 1996] and, under this EMSP project, methanotrophic bacteria.[Hurst *et al.*, 1998a, b] Mass spectrometric detection is improved by concentrating on designing PCR products that are smaller than, but retain equivalent information to, PCR products conventionally detected using gel electrophoresis. This method has the potential for detecting DNA signature molecules much more rapidly and with more specificity than existing techniques. It also has the potential to be automated.

This project made substantial progress along a path toward more rapid analysis of microbial PCR products, using MALDI-MS instead of traditional gel electrophoretic or hybridization methods. The long-term payoff of the described research would be a much higher-throughput analytical method with enhanced accuracy, providing a valuable tool for microbiologists in evaluating and modifying environmental microbial populations. Ultimately, this work could lead to the identification of a suite of diagnostic DNA sequences that can be measured to assess bioremediation processes. These technologies could then be incorporated into a field-portable mass spectrometer so that these parameters can be determined on site. Although construction of such a device was outside the scope of this project, such a system could be used for initial laboratory-based site characterization and also to monitor bioremediation processes during their implementation.

Methods and Results

In this EMSP project (for which funding ended in March 2000), we demonstrated proof of principle for the idea of using MALDI-MS as a readout technique for gene probe assays (PCR in particular) of microbes relevant to bioremediation. Professor Lidstrom’s group at the University of Washington designed special PCR products that are tailored for detection by MALDI-MS. The importance of these PCR products is that they are small enough to allow robust detection

by MALDI-MS, yet contain equivalent information to the larger PCR products that are typically designed for conventional detection techniques. At ORNL, we have used these tailored products as a model system in developing a streamlined MALDI-MS protocol. We have characterized these products and examined the effects of interferences on our protocol. Aspects of this protocol include a rapid method for preparing PCR products for MALDI-MS analysis, parallel implementation of this purification, and automated MALDI-MS data acquisition. We have achieved advances in figures of merit for MALDI-MS analysis of PCR products, including useable mass range, resolution, and reproducibility.

Prof. Lidstrom's group at the University of Washington developed molecular techniques for analyzing natural populations of methanotrophic bacteria. The initial study focused on Lake Washington sediments, a habitat studied in detail by the Lidstrom laboratory. A sequence database was developed for methanotrophs in Lake Washington sediment, both from isolated strains and from environmental clone banks for three sets of diagnostic genes, 16S rRNA, *pmoA* (encoding a subunit of the particulate methane monooxygenase) and *mmoX* (encoding a subunit of the soluble methane monooxygenase). This database was used to design PCR primers and hybridization probes specific for these sets of diagnostic genes that will detect the entire range of these genes in known methanotrophs. These molecular tools were used to analyze the natural populations of methanotrophs in Lake Washington sediments by conventional hybridization techniques. Part of this work has been published (Costello and Lidstrom 1999). A major surprise from this work was the finding that a significant segment of the natural population are sMMO-containing *Methylomonas* strains. These strains are of interest because they have the potential to carry out high rate degradation of trichloroethylene (TCE) and are more easily enriched than the classical *Methylosinus* strains that are normally the target of methane-enhanced bioremediation protocols. New primers have been designed to detect this group of organisms.

This traditional analysis was performed in concert with new analysis methods using MALDI-MS. The first step was to use the new sequence database to design PCR primers to amplify 56- and 99-base pair regions from the *pmoA* gene that encodes the particulate methane monooxygenase enzyme. These relatively short PCR products are specific to two major groups of methanotrophs, type I (represented by *Methylomicrobium albus* BG8) and type II (represented by *Methylosinus trichosporium* OB3b). The UW group shared primer sequences, bacterial genomic DNA, and information on PCR conditions with the ORNL group, allowing the PCR products to be generated at ORNL as needed for mass spectrometry experiments.

Using these products as a model, we developed a robust purification and MALDI-MS protocol that allows detection of these products from a single 25 μ L PCR preparation [Hurst *et al.*, 1998a, b; Buchanan *et al.*, 1998]. Figure 1 compares MALDI-MS spectra of the type II methanotroph PCR product from the *pmoA* gene, before and after the reverse-phase purification. The purified product shows a higher signal-to-noise ratio and improved resolution (narrower peaks). Individual components of the PCR product are resolved in the purified product; these may correspond to sequence heterogeneity and non-templated addition of an extra base by the polymerase enzyme. They do not appear to be MALDI artifacts such as fragmentation or adduction, as illustrated by Figure 2. This spectrum was obtained from a mixture of a synthetic DNA 50-mer and a purified type II *pmoA* PCR product. The peak due to the synthetic 50-mer does not show significant fragmentation or adduction, yet the PCR product shows several peaks. It is thus most likely that the several peaks observed for the PCR product are actually produced in the reaction, rather than being MALDI artifacts. It should be noted that while PCR produces double-stranded DNA, the MALDI process generally "melts" the product into the single

strands, each of which will have its own base composition and molecular mass which, if sufficiently different, could be resolved in the MALDI-MS spectrum.

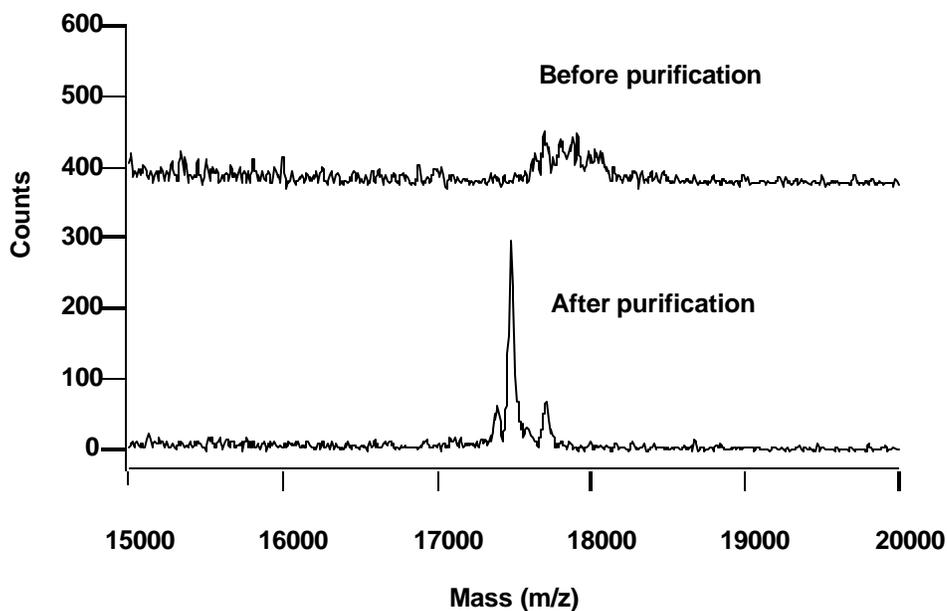


Figure 1. Comparison of MALDI-MS spectra before and after rapid reverse-phase purification of 56-mer type II methanotroph PCR product.

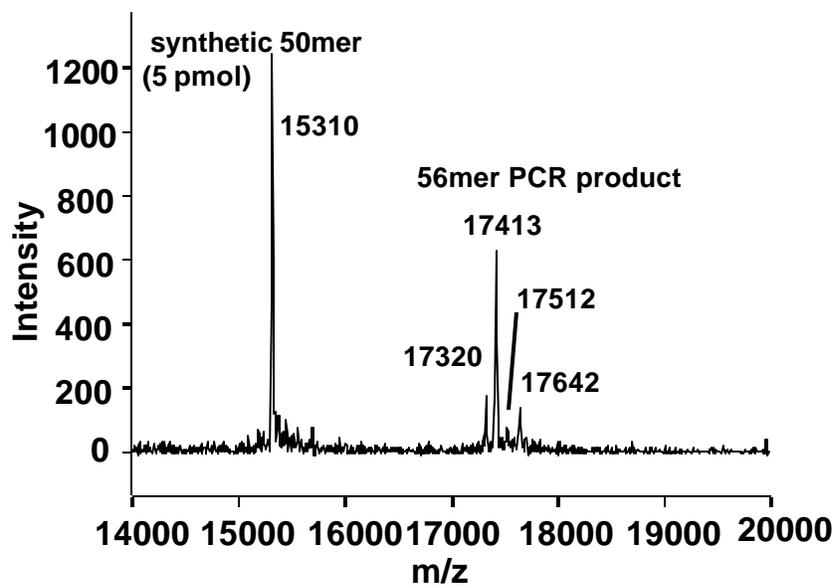


Figure 2. Negative-ion MALDI-MS mass spectrum of 5 picomoles of a synthetic 50-mer (calculated mass 15315 Da) and a type II methanotroph *pmoA* PCR product.

Having developed conditions for MALDI-MS measurement of the pure PCR products, we then explored the effects of various potential interferences on the assays.

We demonstrated first that no products from cross amplification reactions were detected for the two methanotroph assays developed at the University of Washington. Figures 3 and 4 illustrate this result for the type II and type I assays, respectively. PCR reactions were prepared using type II PCR primers, combined with either type II genomic DNA (which should yield an amplification product), type I genomic DNA (which should result in no amplification), or no added bacterial genomic DNA (should give no product; a “blank”). Figure 3 shows that a product at the expected m/z for the type II product ($\sim 17,400$ Da) was detected only when type II DNA was present; no cross-amplification or contamination of the reactions was observed. Similarly, Figure 4 shows that a type I product was observed from a PCR that contained type I primers and type I chromosomal DNA, but no amplification occurred with type I primers combined with type II chromosomal DNA or a blank. As Figures 3 and 4 show, the primer pairs developed for two closely-related bacteria do not cross-amplify. Although this simple demonstration does not preclude the possibility of false positives in other cases, the specificity of PCR primer pairs combined with the ability to measure the size of the resulting PCR product greatly diminishes the possibility that our approach would falsely indicate the presence of a targeted gene sequence due to unintended amplification of interfering DNA. Obviously, many more species would be present in a real sample, and further work would be required to evaluate the utility of the assay in such a situation.

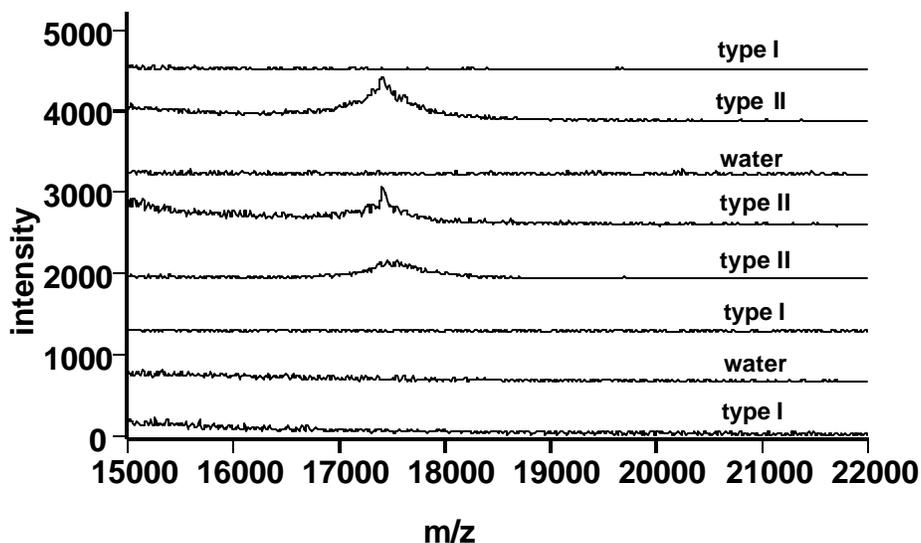


Figure 3. Negative-ion MALDI-MS mass spectra obtained from PCR reactions using the type II primer set with either type I or type II chromosomal DNA or no added DNA (“water”).

Groundwater, soil, or any other subsurface sample will harbor a rich mixture of different microbes, and the potential for this diverse microbial population to contain species that could interfere with a PCR designed to probe a single type of gene must be considered. In addition to

the issue of false positives addressed by the experiments summarized in Figures 3 and 4, DNA from other organisms can decrease amplification efficiency of the targeted DNA by, for example, complexing Mg^{2+} needed by the polymerase enzyme. To determine whether “interfering” DNA could negatively impact our *pmoA* assays, we performed PCR on mixtures of genomic DNA from *Methylosinus trichosporium* OB3b and *E. coli*, the latter acting as the interference DNA in the assay. Figure 5 shows MALDI mass spectra for 10:1, 1:1, and 1:10 ratios of target to interfering DNA used in the PCR. The PCR product from the targeted organism is observed in all cases, indicating that the effect of the interference DNA was negligible under these conditions.

To investigate the detection sensitivity of our method, we varied the starting amount of bacterial DNA carried through the entire process of PCR amplification, purification, and MALDI-MS detection. Figure 6 shows MALDI spectra of the 56-mer PCR product from *Methylosinus trichosporium* OB3b, for different starting amounts of genomic bacterial DNA in the PCR. MALDI signal can be detected from a PCR preparation that uses as little as 1.3 ng of bacterial genomic DNA, corresponding to approximately 10^5 - 10^6 target molecules (assuming a similar genome size for *M. trichosporium* and *E. coli* bacteria [Innis and Gelfand, 1990]). This result could probably be extended to smaller amounts of starting material by optimizing PCR conditions for low numbers of targets.

As described above in the discussion of Figure 1, we interfaced PCR with MALDI-MS detection using a simple solid-phase extraction procedure to remove reagents that are necessary for the PCR, but that subsequently interfere with MALDI-MS size measurement of the amplified DNA. To address the issue of applying our results to large numbers of samples that would be encountered in evaluating or monitoring the bioremediation potential of a site, we scaled up this PCR purification method to a 96-well microtiter plate format, and made progress in automating MALDI-MS data acquisition for up to 100 samples [Weaver *et al.*, 1998] using a commercial MALDI-MS instrument (PerSeptive Biosystems Voyager DE) acquired with EMSP and DOE OBER funding. Figure 7 shows sixteen MALDI-MS spectra obtained from a 96-well purification of the 56-mer *pmoA* PCR product from the type II methanotroph. A number of PCR's were pooled for this purification experiment to reduce the variation due to amplification differences, allowing us to concentrate on well-to-well differences in the purification and in the automated MALDI data acquisition. Some positions of the 96-well purification device were loaded with a synthetic DNA 50-mer for calibration and quality assurance purposes. The purification was performed on all 96 samples in parallel, so that the entire purification required only approximately 30 minutes. This time could be reduced further with robotic pipetting. Recovery of the PCR product, measured using an intercalating fluorescent dye, is 60-70% over the range of product sizes from 50 (primer dimer) up to 200 base pairs. After purification, each sample was mixed with MALDI matrix solution and transferred to a spot on the 100-position sample plate. The automated data acquisition capabilities of the mass spectrometer were used, and MALDI-MS spectra of the samples were obtained without user intervention. This data acquisition required approximately 3 hours. Note in Figure 7 that the 56-mer PCR product is detected with quite good signal-to-noise. Typically, ~90% of the MALDI spots from a 96-well purification yielded robust 56-mer signal in the automated MALDI-MS data acquisition mode, requiring the remaining 10% of the spots to be analyzed manually. While the resolution (related to the narrowness of the peaks) is somewhat variable, the selectivity afforded by the PCR (*i.e.*, products of only a single size are likely to be produced) does not require all spectra to be optimally resolved in order to obtain useful information on the presence or absence of bacterial genes relevant to bioremediation. Our rapid, parallel purification technique is thus an effective interface between PCR and MALDI-MS. A manuscript describing the 96-well purification and automated MALDI data acquisition is in preparation.

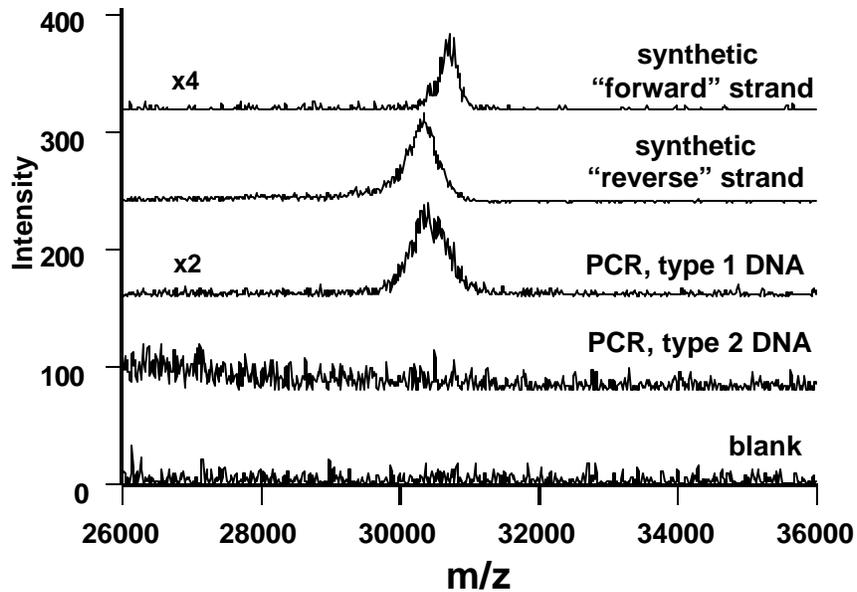


Figure 4. Negative-ion MALDI-MS mass spectra obtained from (upper two spectra) single-stranded synthetic 99-mers and (lower three spectra) PCR reactions using type I primers with either type I or type II chromosomal DNA or no added DNA.

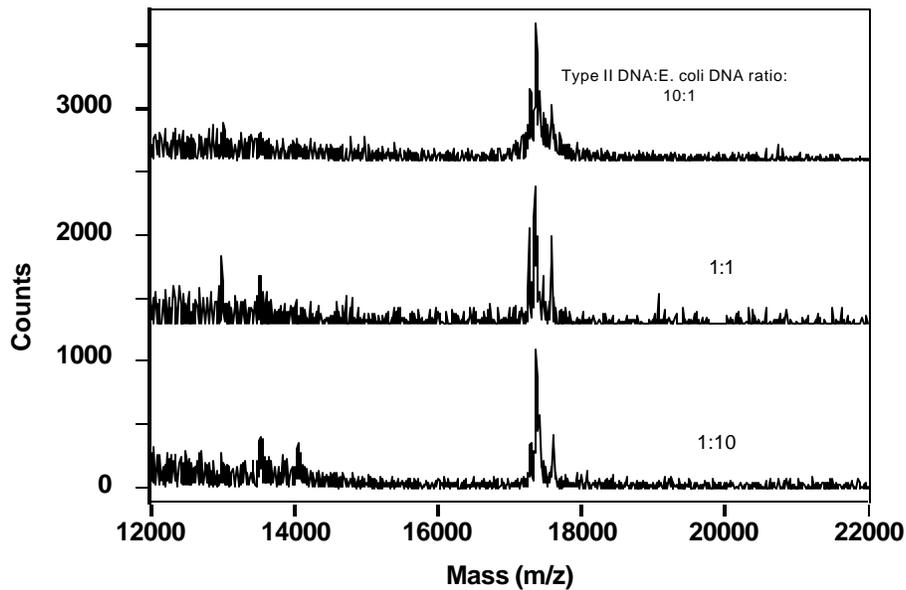


Figure 5. MALDI detection of methanotroph PCR product amplified in the presence of *E. coli* DNA.

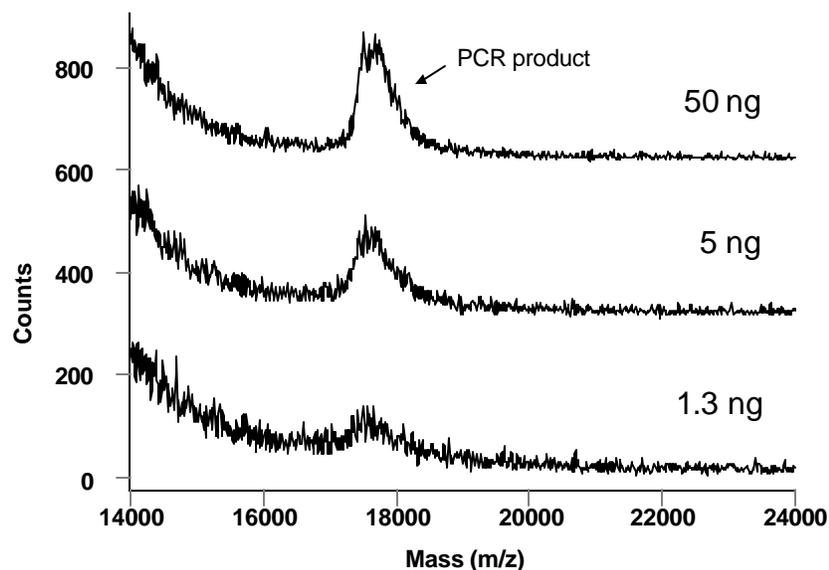


Figure 6. MALDI detection of 56-mer product from PCR amplifications performed using different starting amounts (shown in the figure) of bacterial genomic DNA. Resolution was not optimized in this case.

Because of the large number of existing PCR assays that were designed for conventional detection techniques and amplify target regions in the 100-500 base pair size range, MALDI-MS would be more generally useful if it were applicable to PCR products larger than the specially-designed 56-mer and 99-mer described above. Figure 8 shows MALDI spectra of a 50-mer, a 100-mer, and a 200-mer PCR product, illustrating that while we can indeed detect fairly large products, there remains a need for further work. Note that the peaks increase in breadth with increasing molecular mass. This means that it is more difficult to resolve closely-spaced products at the larger size range. The signal to noise ratio (S/N) also decreases with increasing PCR product size. Thus, although it is possible to detect at least up to a 200-mer in a semi-routine fashion, and 500-600 mers have been reported [Tang *et al.*, 1994; Liu *et al.*, 1995], it is still easier to analyze sizes below this limit at present, such as those developed by Dr. Lidstrom's group.

One goal in our original 1996 proposal was to achieve single-base resolution of DNA in the 100-mer size range. While this is not yet a routine achievement, we have come very close to realizing this goal. Figure 9 shows the MALDI spectrum from a mixture of single-stranded synthetic DNA oligonucleotides of 99 and 101 base lengths, *i.e.*, a 2-base difference. The two observed peaks are easily distinguished, although not baseline resolved. Figure 9 suggests that we are quite close to being able to distinguish a 1-base difference at this size.

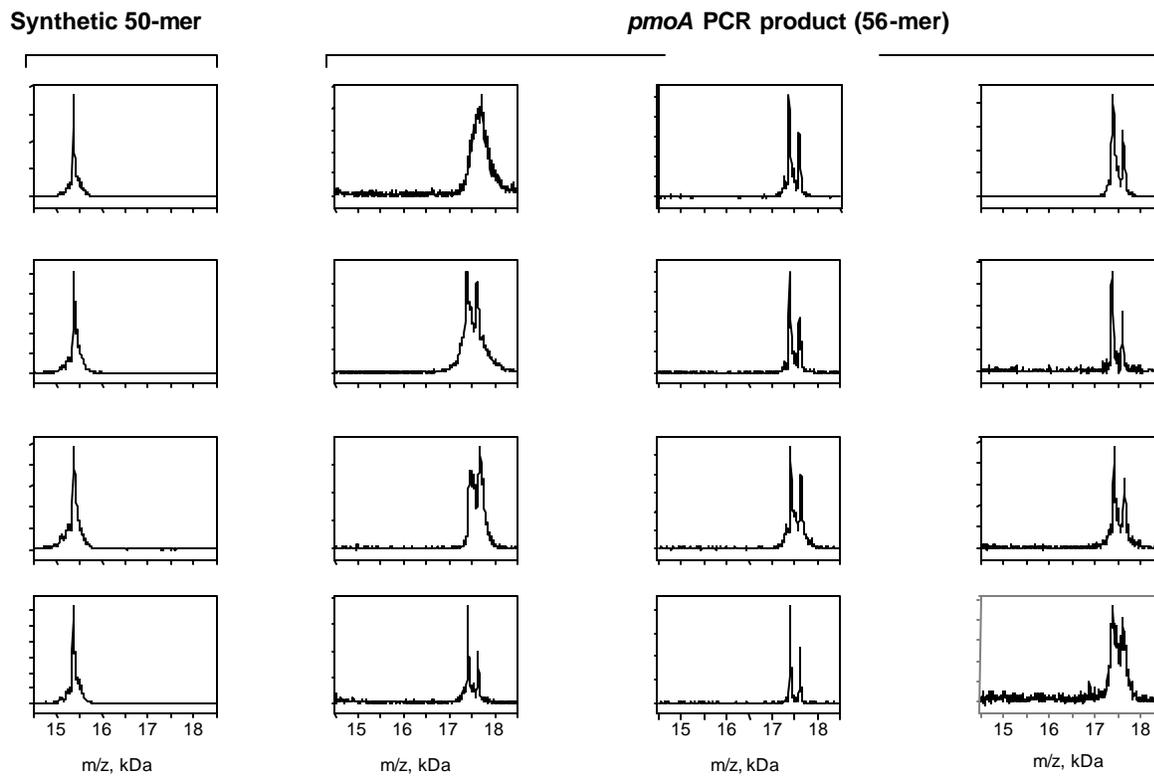


Figure 7. 16 (of 96 total) MALDI spectra obtained automatically following 96-well PCR product purification. (The remaining 80 spectra are omitted due to space considerations.)

While higher throughput has been achieved for smaller *pmoA* PCR products such as the 56-mer shown in Figure 7, a good deal of patience and operator skill are still required to achieve results such as those shown in Figure 9. Therefore, although we have made progress in this area, there is a need to reduce further the “art” associated with MALDI, especially for DNA that contains 100 bases or more. One current problem is that MALDI matrices for DNA typically yield an uneven deposition of DNA-doped matrix crystals on the periphery of the dried sample spot, necessitating a tedious search for “sweet spots” with the laser. For automated, high throughput MALDI-MS analysis of PCR products, it is important to obtain homogeneous MALDI spots that yield good signal from any location on the spot. We have developed a procedure [Kim *et al.*, 1999] using a two-layer substrate of linear polyacrylamide (LPA) or poly(ethylene oxide) and Nafion to obtain good MALDI spectra from any part of a MALDI spot prepared with a mixed matrix containing 3-hydroxypicolinic acid, [Wu *et al.*, 1993] picolinic acid, and ammonium citrate. [Tang *et al.*, 1994] For these experiments, MALDI spots containing a synthetic 20-mer DNA labeled with a covalently-attached fluorescent dye (HEX) were imaged by a fluorescence microscope equipped with a CCD camera, and subsequently subjected to MALDI-MS analysis. While DNA/matrix crystals form only at the spot’s rim in the absence of a polymeric substrate (Figure 10 A), the use of an LPA-Nafion substrate enhances the formation of DNA-doped matrix crystals in the interior of the MALDI spot, as Figure 10 B clearly shows. Not only is the spot

more uniform, but, more importantly, good MALDI spectra were obtained from most locations on the sample spot shown in Figure 10 B. For a number of different MALDI sample spots, we obtained MALDI spectra at ≥ 14 locations for the laser within each spot. A positive hit was scored if the signal-to-noise ratio for the MALDI spectrum of the DNA was ≥ 4 . For bare metal sample plates, the hit rate was 52%, with a standard deviation of 16% determined by analyzing 6 separate sample spots. For an LPA/Nafion substrate, the hit rate was $84 \pm 3\%$ (12 separate sample spots). The hit rate, indicating within-spot ability to locate the signal, was thus substantially higher for the LPA/Nafion substrate. Also, the between-spot variation was reduced for the samples prepared on the LPA/Nafion substrates. We evaluated other potential substrate materials, including poly(ethyleneimine), poly(decyl acrylate), poly(acrylic acid), and methyl cellulose, none of which have yielded satisfactory results. A manuscript describing these results is currently in preparation.

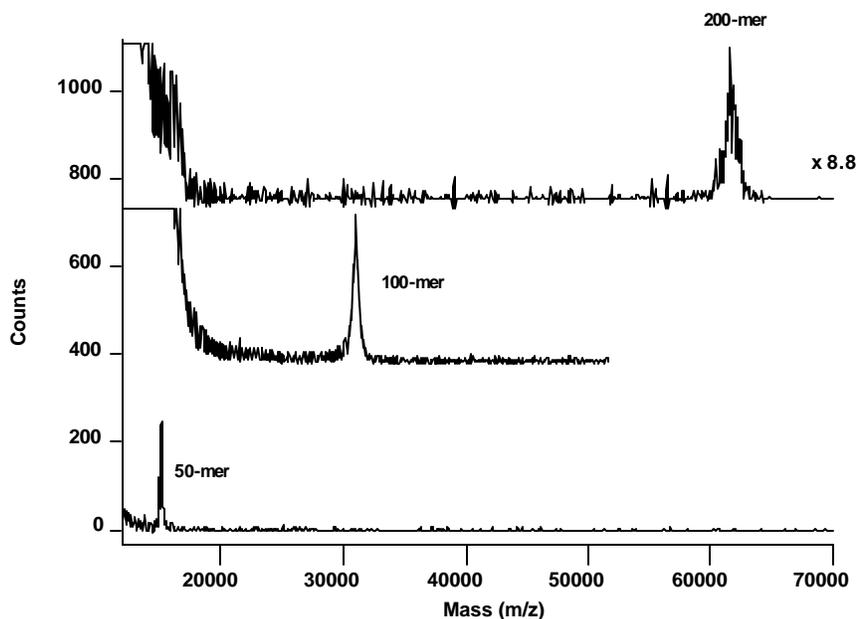


Figure 8. MALDI spectra of 50-mer, 100-mer, and 200-mer PCR products.

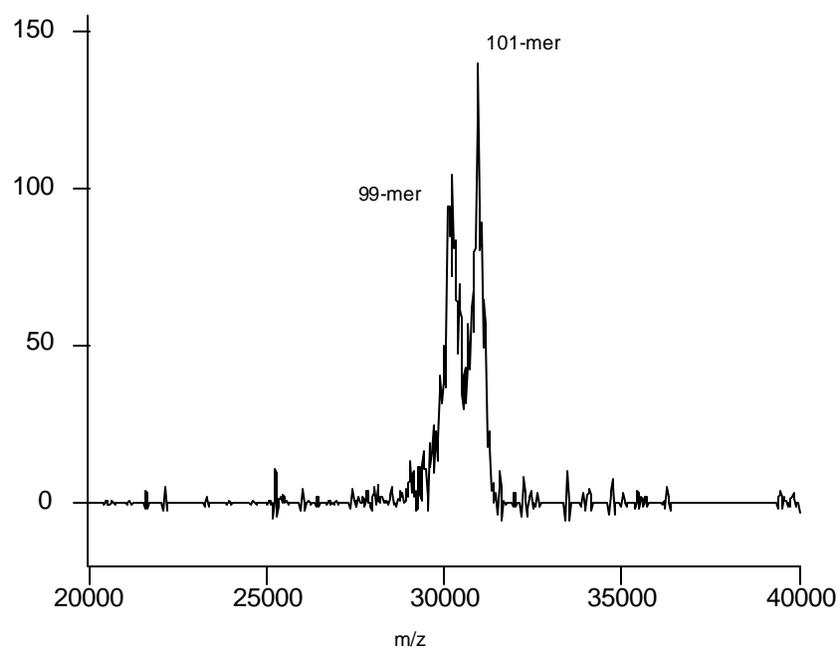


Figure 9. MALDI spectra showing resolution of a 99-mer from a 101-mer.

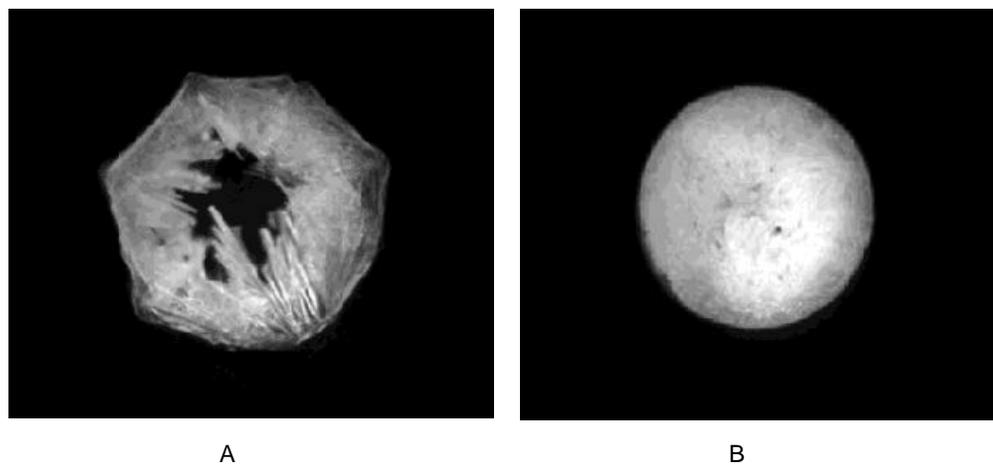


Figure 10. Fluorescence micrographs of DNA in MALDI matrix spots. A: conventional MALDI sample spot; B: MALDI spot deposited on an LPA/Nafion substrate.

Relevance, Impact and Technology Transfer

a. How does this new scientific knowledge focus on critical DOE environmental management problems?

DNAPLs such as chloroform, carbon tetrachloride, trichlorethylene and perchloroethylene represent a significant fraction of the pollution legacy remaining from past operations at many DOE and industrial sites. As these chemicals represent a significant human health risk and have potential for migrating into drinking water supplies under the influence of geological processes, characterization and cleanup of DNAPLs is an important part of the mission of EM, in particular the Subsurface Contaminants Focus Area (SCFA), and its DNAPL product line. [Phillips, 1999]

The SCFA Multi-Year Program Plan, FY 2000-2004, addresses this issue [Wright *et al.*, 1999]:

“The capability to destroy contaminants *in situ* is a preferred method of remediation because it reduces the risk both to the environment and to the public and is typically less expensive. These techniques produce much lower levels of secondary waste, thus reducing future waste legacies.

Access to contaminants in the diverse geologic settings across the complex is the greatest challenge to *in situ* destruction. Dense, Non-Aqueous Phase Liquid (DNAPL) concentrations in the sediment below the water table, if not destroyed, will continue to contaminate the aquifer for years. Promising technologies to define and treat these areas of high concentration levels in complex hydrogeologic conditions are being demonstrated by the SCFA.”

Because it can be applied *in situ*, bioremediation is thus an important weapon in the DOE arsenal for remediation of DNAPLs. Further information about the application of bioremediation at DOE sites can be found in the SCFA “Rainbow Book” [U.S. DOE, 1996].

The knowledge gained as a result of this project provides proof of principle for a new method for profiling microbial populations at DOE sites being considered for *in-situ* bioremediation of organics or metals.

b. How will the new scientific knowledge that is generated by this project improve technologies and cleanup approaches to significantly reduce future costs, schedules, and risks and meet DOE compliance requirements?

One aspect of *in-situ* bioremediation where improvements are needed is characterizing and monitoring the microbial populations that actually perform the degradation of pollutants. [Foreman, 1999; U.S. DOE, 1996] In order to develop bioremediation into a reliable and cost-effective treatment strategy for cleanup of DNAPLs, a rapid screening tool is needed for metabolic and genetic potential of the indigenous microbial population for destroying the particular pollutants. A readily available, broad-brush assessment of key traits involved in biodegradation of DNAPLs would reduce uncertainty in effectiveness and cost related to bioremediation alternatives, and in the optimization of a cleanup system once it was implemented. Faster and more convenient microbial characterization techniques would enable the selection of the bioremediation strategy most suited to a site, reducing the need for costly pump and treat, soil excavation, or other remediation technologies that generate secondary

waste products, expose workers to pollutants, or simply cannot be applied in unfavorable geological circumstances.

Because of the potential cost benefits associated with *in-situ* bioremediation, this research could reduce future costs by allowing broader and speedier implementation and monitoring of *in-situ* bioremediation in cases where it is not presently applicable.

c. To what extent does the new scientific knowledge bridge the gap between broad fundamental research that has wide-ranging applications and the timeliness to meet needs-driven applied technology development?

The broad fundamental research on which this project draws lies in two areas: 1. Molecular biology of microbes and the sequencing of their genomes and identification of relevant genes that degrade pollutants, and 2. Developments in mass spectrometry that have allowed analysis of larger biomolecules such as the PCR-amplified regions of environmentally relevant microbial genes. The needs-driven technology development issue addressed by this project is microbial characterization for assessing the *in situ* bioremediation potential of a polluted site. We have narrowed the gap between broad fundamental knowledge and needs-driven technology development by demonstrating the detection of microbial genes that degrade TCE using molecular biology and mass spectrometry-based techniques.

d. What is the project's impact on individuals, laboratories, departments, and institutions? Will results be used? If so, how will they be used, by whom, and when?

At ORNL, the knowledge gained as a result of this project has broadened the knowledge and experience base of the Organic and Biological Mass Spectrometry group in the application of mass spectrometry to DNA. We are as a result of this project able to carry out PCR amplification of desired products, both for specific applications and for model systems. The ability to desalt and purify PCR products and other small DNA molecules is also valuable in on-going research in the group. Because it is the genetic material common to all organisms, the knowledge gained in this project will be helpful in collaborations with other life scientists in the future.

Two postgraduate researchers were involved in the project at ORNL; Dr. Yongseong Kim, now assistant professor in the Division of Chemistry and Chemical Engineering at Kyungnam University in Masan, South Korea, and Kristal Weaver, presently senior research assistant at the Human Immunology and Cancer Research Program, University of Tennessee--Knoxville.

At the University of Washington, two graduate students have been partially supported in their Ph.D. research by this project. Dr. Andria Costello is now an assistant professor at Syracuse University, in the Department of Civil & Environmental Engineering. Ann Auman is currently completing her Ph.D. studies in Prof. Lidstrom's laboratory.

e. Are larger scale trials warranted? What difference has the project made? Now that the project is complete, what new capacity, equipment or expertise has been developed?

Because of the encouraging preliminary results obtained in this project, we have submitted a renewal proposal to the EMSP to continue this work. The scope of the work in the renewal period would involve development of a wider array of microbial gene probes tailored for mass spectrometry detection, as well as demonstration of the technology on groundwater samples from Lake Washington and DOE sites.

f. How have the scientific capabilities of collaborating scientists been improved?

The interdisciplinary nature of the work, involving molecular biology and analytical chemistry, has broadened the knowledge base of both groups.

g. How has this research advanced our understanding in the area?

A DNA sequence database was developed for methanotrophs in Lake Washington sediment. It was found that a significant segment of the natural population are sMMO-containing *Methylomonas* strains. These strains are of interest because they have the potential to carry out high rate degradation of trichloroethylene (TCE) and are more easily enriched than the classical *Methylosinus* strains that are normally the target of methane-enhanced bioremediation protocols.

Improved methods for mass spectrometric analysis of PCR products were developed. This involved optimization of a purification procedure, which was implemented in a rapid, parallel fashion, and development of a sample substrate that shows promise for producing more homogeneous, and therefore more easily analyzed, DNA/matrix mixtures for MALDI-MS.

h. What additional scientific or other hurdles must be overcome before the results of this project can be successfully applied to DOE Environmental Management problems?

The two major hurdles remaining before this project could be applied to DOE Environmental Management problems are (1) further improvements in robustness for the mass spectrometry detection of PCR amplification products, and (2) development of "mass spectrometry friendly" gene probes for a broader range of microbial metabolic functions relevant to bioremediation. These two hurdles are addressed in our renewal proposal, currently under review.

i. Have any other government agencies or private enterprises expressed interest in the project? Please provide contact information.

No.

Project Productivity

Initially, we proposed two fully-funded projects--one at ORNL, and the other at the University of Washington. Only the ORNL proposal was funded in 1996. However, because of the importance of the work proposed by Prof. Lidstrom's group to the success of the project, we supported a small subset of the proposed UW research through a subcontract. For this reason, the goals originally proposed were not fully achieved. To be more specific, the original pair of proposals outlined work to identify products of DNAPL metabolism using electrospray/ion trap mass spectrometry; this work was not performed due to funding at a lower level than requested originally. However, we did demonstrate proof of principle for the mass spectrometric detection of gene probe assays for microbial genes relevant to bioremediation.

Personnel Supported

Oak Ridge National Laboratory:

Michelle V. Buchanan, PI Associate Division Director, Life Sciences Division (LSD)

Gregory B. Hurst	Research Staff, Chemical and Analytical Sciences Division (CASD)
David P. Allison	Research Staff, LSD
Phillip F. Britt	Research Staff, CASD
Mitchel J. Doktycz	Research Staff, LSD
Yongseong Kim	Postdoctoral Research Program, ORISE
Kristal Weaver	Postgraduate Research Program, Oak Ridge Institute for Science and Education (ORISE)
University of Washington:	
Mary E. Lidstrom	Frank Jungers Professor of Chemical Engineering and Professor of Microbiology
Andria M. Costello	Ph.D. Student
Ann Auman	Ph.D. Student

Publications

Articles stemming from the research which were:

a. Published in peer-reviewed journals and books.

G.B. Hurst, K. Weaver, M.J. Doktycz, M.V. Buchanan, A.M. Costello and M.E. Lidstrom. "MALDI-TOF Analysis of Polymerase Chain Reaction Products from Methanotrophic Bacteria," *Anal. Chem.* **1998**, *70*, 2693-2698. Results were summarized above in Section 5.

A.M. Costello and M.E. Lidstrom, "Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments," *Appl. Env. Microbiol.* **1999**, *65*, 5066-5073. Results were summarized above in Section 5.

b. Published in unreviewed publications (proceedings, technical reports, etc.).

G.B. Hurst, K. Weaver, M.V. Buchanan and M.J. Doktycz, "Analysis of PCR products using delayed-extraction MALDI-TOF," Proceedings of the 45th ASMS Conference on Mass Spectrometry and Allied Topics, Palm Springs CA, June 1-5, 1997, p. 843.

G.B. Hurst, K. Weaver, M.J. Doktycz, M.V. Buchanan, A. Costello, and M.E. Lidstrom, "Identification of Methanotrophic Bacteria Using the Polymerase Chain Reaction with MALDI-TOF Detection," Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics, Orlando FL, May 31-June 4, 1998, p. 1202.

K. Weaver, M.J. Doktycz, P.F. Britt, G.B. Hurst, and M.V. Buchanan, "96-Well Microtiter-Format Purification of DNA for MALDI-TOF Analysis," Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics, Orlando FL, May 31-June 4, 1998, p. 60.

Y. Kim, G.B. Hurst, M.J. Doktycz and M.V. Buchanan, "Improved Spot Homogeneity for DNA MALDI Matrices," Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, Dallas TX, June 13-18, 1999.

c. Accepted/submitted for publication.

Auman, Ann J., Sergei Stolyar, and Mary E. Lidstrom, "Molecular characterization of methanotrophic isolates from freshwater lake sediment," submitted to *Appl. Env. Microbiol.* (2000).

Interactions

a. Participation/presentations at meetings, workshops, conferences, seminars, etc.

G. B. Hurst, K. Weaver, and M.V. Buchanan, "Improved Mass Spectrometric Resolution for PCR Product Size Measurement," presented at The Sixth Department of Energy Contractor and Grantee Workshop of the Human Genome Program, Santa Fe, NM, November 9-13, 1997.

M.V. Buchanan, G.B. Hurst, M.J. Doktycz, P.F. Britt, K. Weaver, M.E. Lidstrom and A.J. Costello, "Monitoring Genetic and Metabolic Potential for *in situ* Bioremediation: Mass Spectrometry," Poster presentation at the DOE Environmental Management Science Program Workshop, Chicago, Ill., July 27-30, 1998.

G.B. Hurst, Y. Kim, K. Weaver and M.V. Buchanan, "PCR Product Size Measurement using MALDI Mass Spectrometry," Poster presentation at the 7th DOE Human Genome Contractor-Grantee Workshop, Oakland, California, January 12-16, 1999.

G.B. Hurst, M.V. Buchanan, M.J. Doktycz, P.F. Britt, Y. Kim, K. Weaver, M.E. Lidstrom, A.M. Costello, A. Auman, "Monitoring Genetic and Metabolic Potential for *in situ* Bioremediation: Mass Spectrometry," Oral presentation at the DOE-ORO EMSP Workshop, Oak Ridge, TN, September 22, 1999.

M.V. Buchanan, G.B. Hurst, M.J. Doktycz, P.F. Britt, K. Weaver, M.E. Lidstrom and A.J. Costello, "Monitoring Genetic and Metabolic Potential for *in situ* Bioremediation: Mass Spectrometry," Poster presentation at the DOE Environmental Management Science Program Workshop 2000, Atlanta, Ga., April 24-28, 2000.

b. Consultative and advisory functions to other laboratories and agencies, especially DOE and other government laboratories.

None.

c. Collaborations

The project itself was collaborative, but did not involve collaboration with others not directly involved in the project.

Transitions

Due to its more fundamental nature in keeping with the EMSP philosophy, this research has not progressed to a point of being applied by personnel involved in DOE or other remediation activities.

Patents

None

Future Work

The two major hurdles remaining before this project could be applied to DOE Environmental Management problems are (1) further improvements in robustness for the mass spectrometry detection of PCR amplification products, and (2) development of "mass spectrometry friendly" gene probes for a broader range of microbial metabolic functions relevant to bioremediation. These two hurdles are addressed in our renewal proposal, currently under review.

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A.M. Costello and M.E. Lidstrom, "Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments," *Appl. Env. Microbiol.* **65**, 5066-5073 (1999).

A.M. Costello, "Molecular Ecology Studies of Methanotrophs in a Freshwater Lake Sediment." Ph.D. Thesis, California Institute of Technology, Pasadena, CA, 1999. Available from UMI, 300 N. Zeeb Rd, Ann Arbor, MI, UMI # 9930380.

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Appendix

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