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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 μL 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.

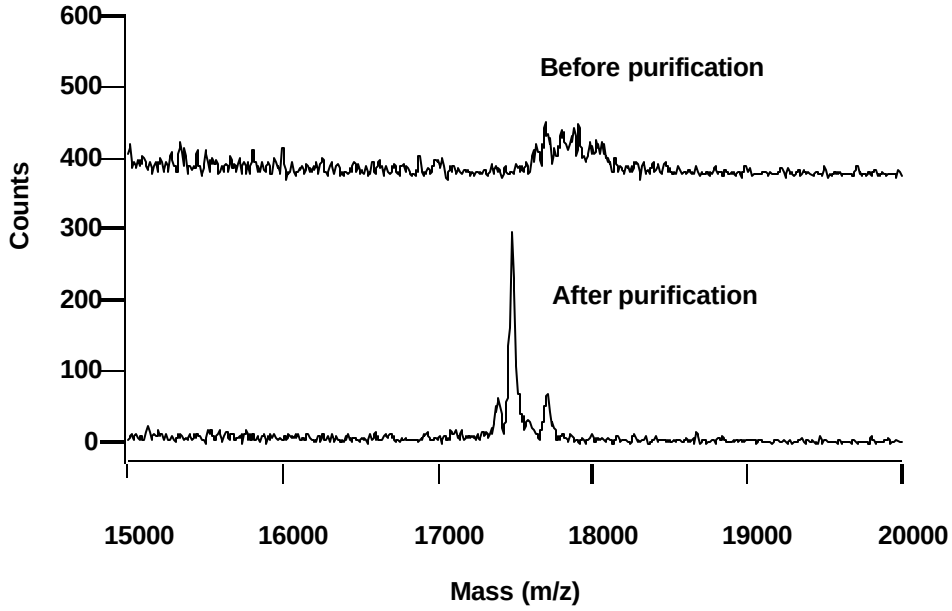

Figure 1. Comparison of MALDI-MS spectra before and after rapid reverse-phase purification of 56-mer type II methanotroph PCR product.

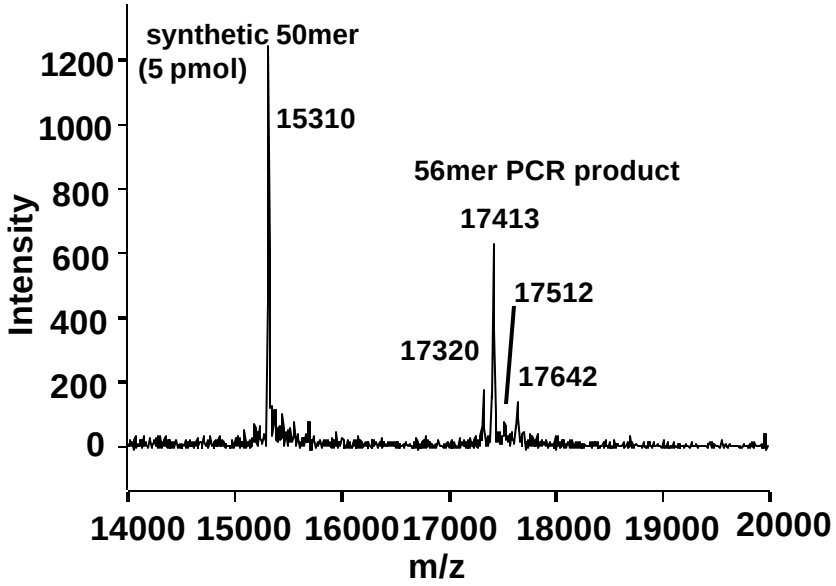

Figure 2. Negative-ion MALDI-MS mass spectrum of 5 picomoles of a synthetic 50-mer (calculated mass 15315 Da) and a type II methanotroph *pmoA* PCR product.

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 (~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.

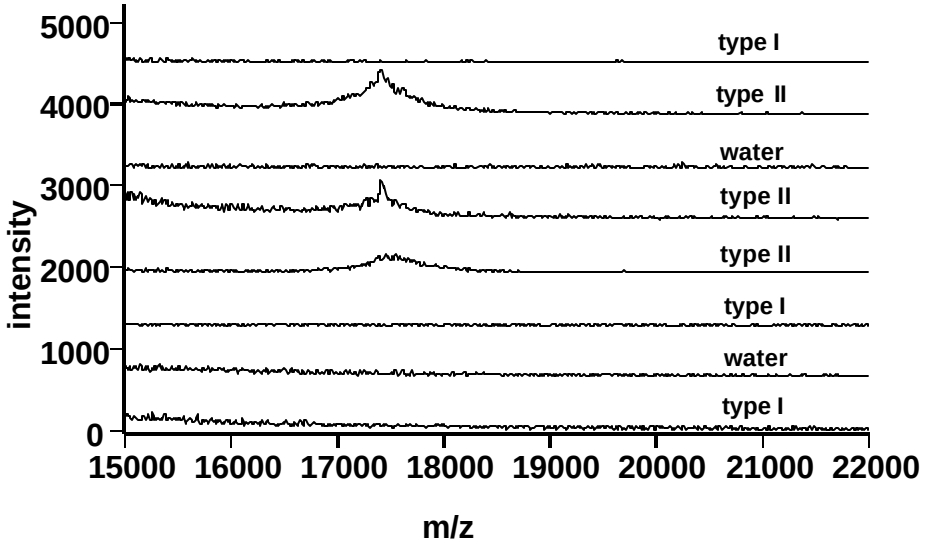

Figure 3. Negative-ion MALDI-MS mass spectra obtained from PCR reactions using the type II primer set with either type I or type II chromosomal DNA or no added DNA ("water").

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.

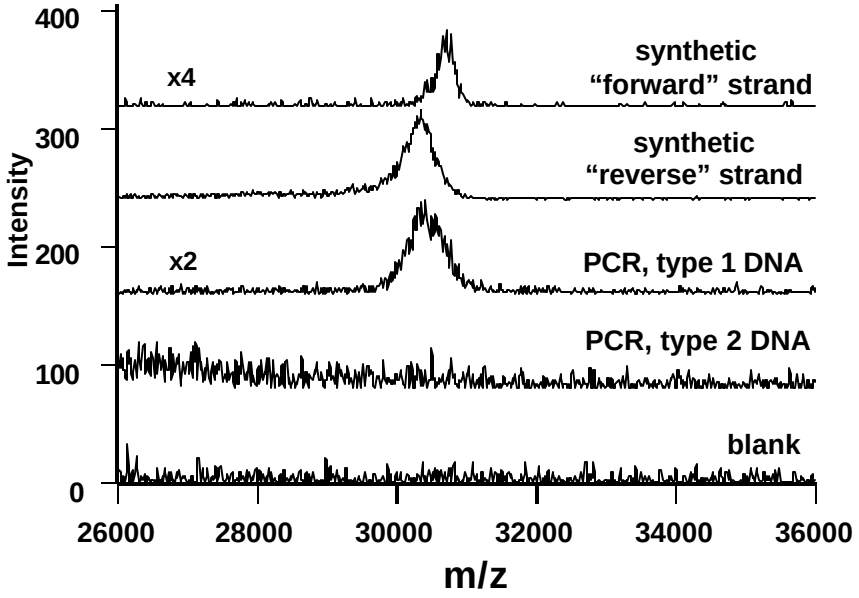

Figure 4. Negative-ion MALDI-MS mass spectra obtained from (upper two spectra) single-stranded synthetic 99-mers and (lower three spectra) PCR reactions using type I primers with either type I or type II chromosomal DNA or no added DNA.

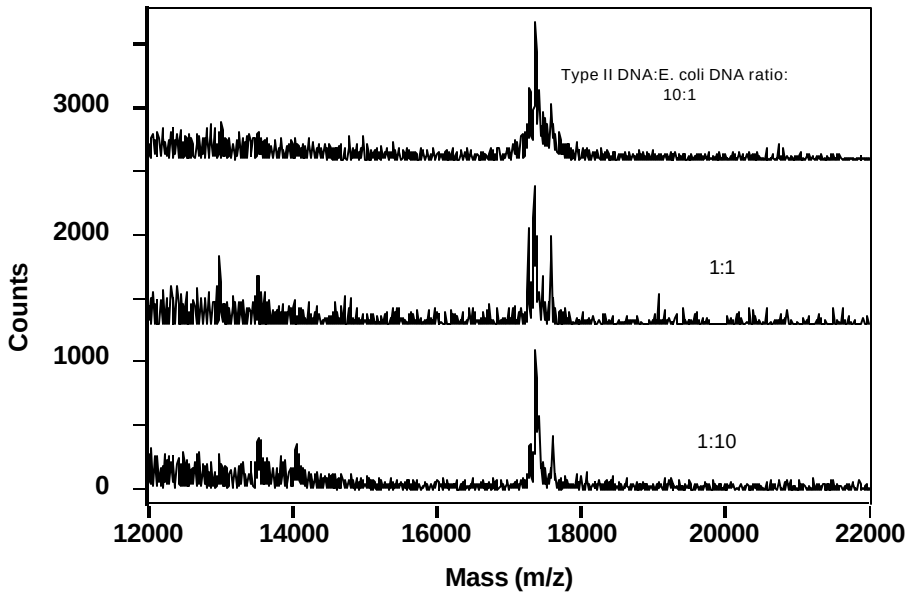

Figure 5. MALDI detection of methanotroph PCR product amplified in the presence of *E. coli* DNA.

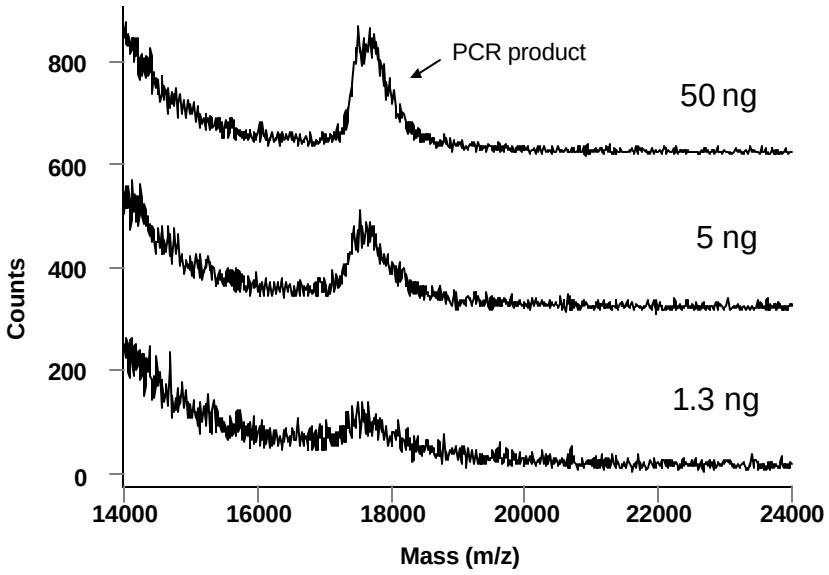

Figure 6. MALDI detection of 56-mer product from PCR amplifications performed using different starting amounts (shown in the figure) of bacterial genomic DNA. Resolution was not optimized in this case.

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 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.

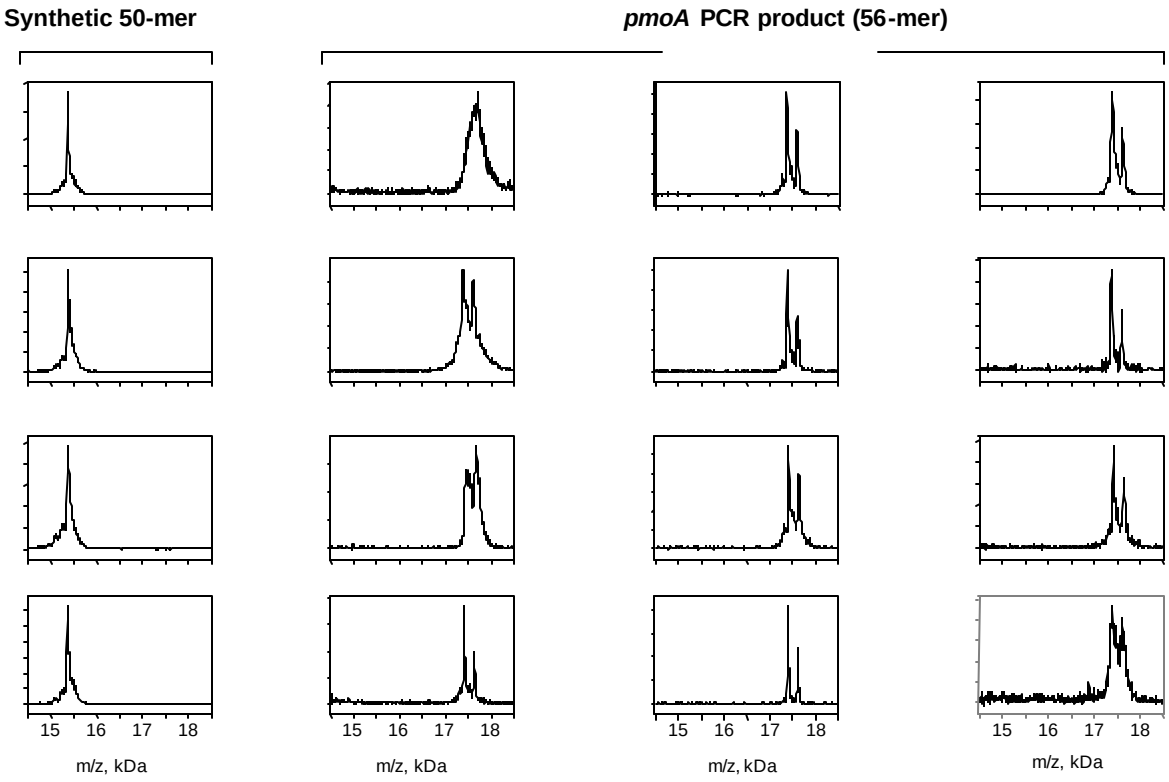

Figure 7. 16 (of 96 total) MALDI spectra obtained automatically following 96-well PCR product purification. (The remaining 80 spectra are omitted due to space considerations.)

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±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.

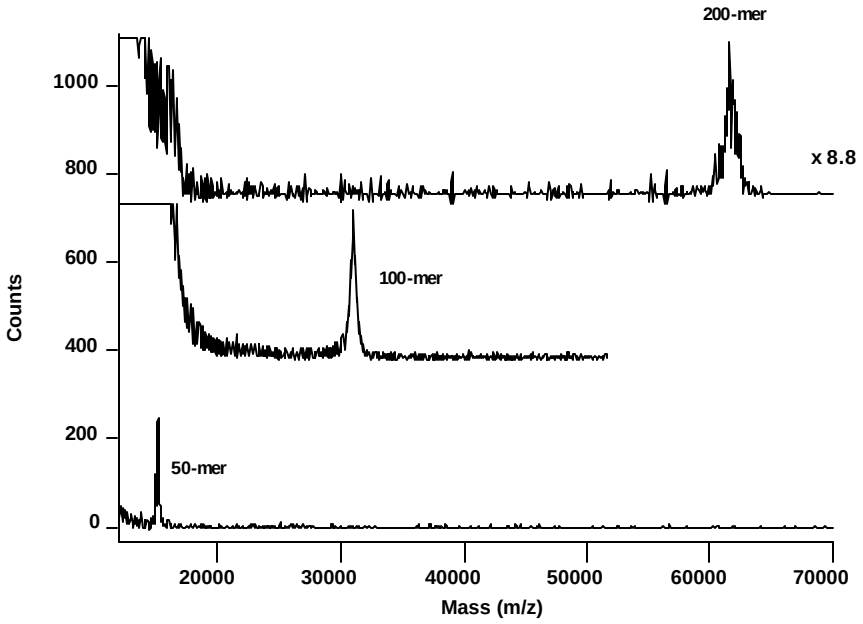

Figure 8. MALDI spectra of 50-mer, 100-mer, and 200-mer PCR products.

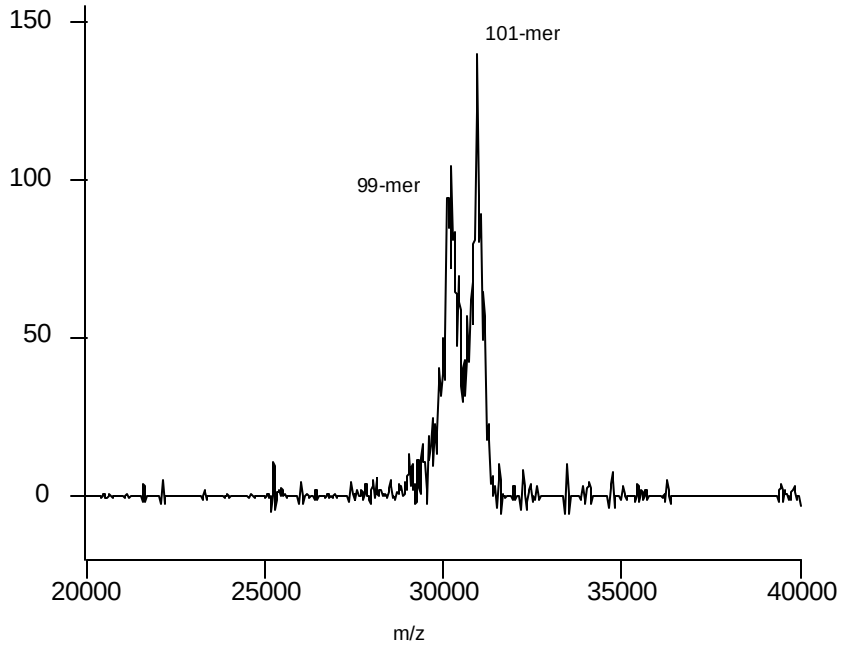

Figure 9. MALDI spectra showing resolution of a 99-mer from a 101-mer.

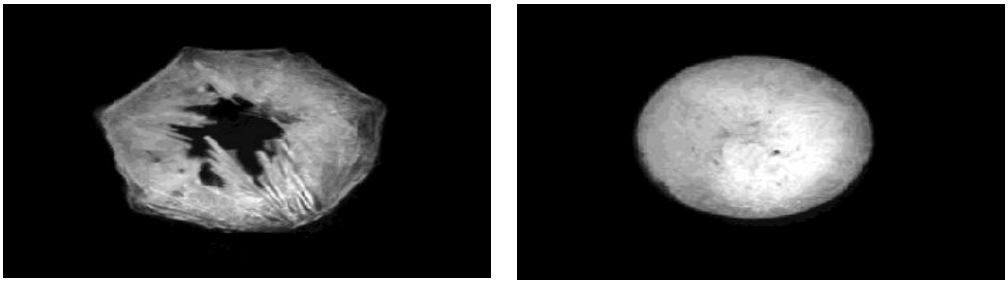

Figure 10. Fluorescence micrographs of DNA in MALDI matrix spots. A: conventional MALDI sample spot; B: MALDI spot deposited on an LPA/Nafion substrate.

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.

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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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M.J. Doktycz, G.B. Hurst, S. Habibi-Goudarzi, S.A. McLuckey, K. Tang, C.H. Chen, M. Uziel, K.B. Jacobsen, R.P. Woychik and M.V. Buchanan, "Analysis of Polymerase Chain Reaction-Amplified DNA Products by Mass Spectrometry Using Matrix-Assisted Laser Desorption and Electrospray: Current Status," *Anal. Biochem*. **230**, 205-214 (1995).

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Appendix

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Molecular Characterization of Functional and Phylogenetic Genes from Natural Populations of Methanotrophs in Lake Sediments

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The 16S rRNA and *pmoA* genes from natural populations of methane-oxidizing bacteria (methanotrophs) were PCR amplified from total community DNA extracted from Lake Washington sediments obtained from the area where peak methane oxidation occurred. Clone libraries were constructed for each of the genes, and approximately 200 clones from each library were analyzed by using restriction fragment length polymorphism (RFLP) and the tetrameric restriction enzymes *Msp*I, *Hae*III, and *Hha*I. The PCR products were grouped based on their RFLP patterns, and representatives of each group were sequenced and analyzed. Studies of the 16S rRNA data obtained indicated that the existing primers did not reveal the total methanotrophic diversity present when these data were compared with pure-culture data obtained from the same environment. New primers specific for methanotrophs belonging to the genera *Methylomonas*, *Methylosinus*, and *Methylocystis* were developed and used to construct more complete clone libraries. Furthermore, a new primer was designed for one of the genes of the particulate methane monooxygenase in methanotrophs, *pmoA*. Phylogenetic analyses of both the 16S rRNA and *pmoA* gene sequences indicated that the new primers should detect these genes over the known diversity in methanotrophs. In addition to these findings, 16S rRNA data obtained in this study were combined with previously described phylogenetic data in order to identify operational taxonomic units that can be used to identify methanotrophs at the genus level.

Methanotrophs are a group of gram-negative bacteria that can grow on methane as the sole source of carbon and energy. They are widespread in nature and have gotten increased attention in the past two decades due to their potential role in the global methane cycle (11) and their ability to cometabolize a number of environmental contaminants (15). The methanotrophs consist of eight recognized genera (3, 5–7) that fall into two major phylogenetic groups, the α subgroup of the class *Proteobacteria* (α-*Proteobacteria*) (which includes the type II methanotrophs) and the γ-*Proteobacteria* (which includes the type I methanotrophs). In addition, a new thermophilic genus, *Methylothermus*, that forms a distinct, deeply branching group within the γ-*Proteobacteria* has recently been described (4).

Traditionally, studies performed with natural populations of methanotrophs have focused on culture-based techniques (15) that may or may not reveal the true diversity in nature (1). More recently, however, researchers have recognized the need for culture-independent analyses of natural methanotrophic populations, and these types of analyses have been facilitated by recent advances in the molecular biology and molecular phylogeny of methanotrophs (16, 24, 28). To aid in these studies, PCR primers targeted to the 16S rRNA genes in methanotrophs have been developed (8, 17). In addition, preliminary work has been carried out to identify primers that detect *pmoA*, one of the genes for the diagnostic enzyme for methanotrophs, the particulate methane monooxygenase (pMMO) (16). These primers also detect *amoA*, which encodes the analogous subunit of the ammonia monooxygenase in nitrifying bacteria (26).

To date, most studies involving non-culture-based analyses of natural populations of methanotrophs have focused on marine and peat bog environments (17, 23, 25). In these studies, nucleic acid-based techniques have been used to obtain information on methanotrophic 16S rRNA and *pmoA* genes. The results of these studies have expanded the known sequence diversity for these genes and have suggested that these environments contain limited methanotroph diversity at the genus level. The environmental sequences obtained from peat environments all cluster with the type II methanotrophs (23, 25), while the two strains from marine and estuarine environments are both type I strains (17, 33).

Workers in our laboratories are interested in investigating natural populations of methanotrophs in freshwater sediments. However, it is not yet clear whether the molecular tools that are currently available detect the full range of in situ methanotroph genera in these environments. Methanotrophs in freshwater sediments are important to the global methane cycle as these environments are predicted to produce an amount of methane equivalent to approximately 40 to 50% of the annual global atmospheric methane flux (11, 18, 31). However, most of this methane never reaches the atmosphere as it is consumed by methanotrophs (18). Some data suggest that freshwater environments may contain greater methanotroph diversity than peat and marine environments since both pure-culture isolation methods and phospholipid fatty acid analyses indicate that a mixture of type I and type II strains is present (2, 9).

Currently, no data concerning the in situ populations of methanotrophs in freshwater environments as determined by using primers specific for methanotroph 16S rRNA or *pmoA*

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TABLE 1. Methanotroph-specific primers used in this study

Primer	Sequence (5'-3')	Target genus or gene	Reference
Mb1007r	CACTCTACGATCTCTCACAG	*Methylobacter Methylomicrobium*	17
Mc1005r	CCGCATCTCTGCAGGAT	*Methylococcus*	17
Mm1007r	CACTCCGCTATCTCTAACAG	*Methylomonas*	17
Ms1020r	CCCTTGCGGAAGGAAGTC	*Methylosinus*	17
Mm835	GCTCCACYACTAAGTTC	*Methylomonas*	This study
Type2b	CATACCGGRCATGTCAAAAGC	*Methylosinus-Methylocystis*	This study
A189gc	GGNGACTGGGACTTCTGG	*pmoA*	16
mb661	CCGGMGCAACGTCYTTACC	*pmoA*	This study

genes are available. In addition, it is not known whether the methanotroph primers that have been described can effectively assess the in situ methanotroph diversity in these habitats. Therefore, the objective of this study was twofold: to develop a database of methanotroph 16S rRNA and *pmoA* sequences for a freshwater sediment and to use this information to develop robust molecular tools for studying in situ methanotrophs in freshwater habitats. The study site chosen was Lake Washington, which we have previously analyzed to determine methanotrophic activities in carbon and oxygen cycling (19, 20).

MATERIALS AND METHODS

Collection of samples. Sediment was collected from a 62-m-deep station in Lake Washington in Seattle, Wash., by using a box core sampler that allowed us to collect relatively undisturbed sediment. Subsections of the box cores were sectioned into 0.5-cm slices to a depth of 5 cm. Samples were kept on ice for approximately 1 to 2 h and were then used or stored at −20°C.

DNA extraction and purification. DNA was extracted from sediment obtained in the area where peak methane oxidation occurred (1a) by using a protocol described by Gray and Herwig (14). The amount of sediment used per extraction procedure was 600 mg. The modifications of the protocol included replacing the Spin-Bind columns with Sephadex G-200 spin columns. The Sephadex G-200 spin columns were constructed by filling a 1-ml syringe with glass wool and approximately 1 to 2 cm of TE-saturated Sephadex G-200. After passage through the column, the DNA was further purified by removing residual humic acids by electrophoresis on a 1% agarose gel and purification with a Qiagen gel extraction kit (Qiagen, Inc.). DNA obtained after this treatment was used in PCR mixtures.

PCR amplification of 16S rRNA and *pmoA* genes. The 16S rRNA genes were PCR amplified from total DNA extracted from sediment by using methanotroph phylogenetic group-specific primers Mb1007, Mc1005, Mm1007, and Ms1020 (17) in conjunction with bacterium-specific primer f27. Furthermore, 16S rRNA primers Mm835 (5' GCTCCACYACTAAGTTC 3') and Type2b (5' CATACCGGRCATGTCAAAAGC 3') were designed by using new and previously described sequences to specifically amplify genes from members of the genus *Methylomonas* and members of the genera *Methylosinus* and *Methylocystis*, respectively (Table 1). These primers were also used in subsequent PCRs with primer f27 to amplify genes from members of the genera *Methylomonas*, *Methylosinus*, and *Methylocystis*. All reactions were carried out in 30-μl (total volume) mixtures containing approximately 100 ng of sediment DNA, 10 pmol of each primer, 1.5 mM Mg^{2+}, Gibco buffer, and 2.5 U of Gibco *Taq* polymerase. The reactions were performed in a Perkin-Elmer model 9600 GeneAmp PCR System thermal cycler by using 25 cycles consisting of 92°C for 1 min, 55°C for 1.5 min (50°C for primer Mm835), and 72°C for 1 min and a final extension step consisting of 72°C for 5 min. In addition, amplification reactions were also performed with primers specific for *pmoA*. To design *pmoA*-specific primers, *pmoA* and *amoA* sequences available from the GenBank database were aligned, and primer mb661 (5' CCGGMGCAACGTCYTTACC 3') was designed (Table 1). Primer mb661 was used in conjunction with primer A189gc (16). Together, primers A189gc and mb661 amplified an approximately 470-bp internal section of *pmoA* and produced strong signals with all of the methanotrophs tested. The methanotrophs tested included pure cultures of *Methylomicrobium album* BG8, *Methylomonas rubra*, *Methylomonas methanica* S1, *Methylococcus capsulatus* Bath, *Methylosinus trichosporium* OB3b, *Methylocystis parvus* OBBP, and the isolates obtained from Lake Washington in this study (see below). The *pmoA* primer pair, primers A189gc and mb661, produced no product with *Nitrosomonas europaea* DNA, as determined in PCRs. In addition, primer mb661 was tested in silico with additional nitrifier *amoA* gene sequences obtained from the GenBank database and exhibited low levels of identity (9- to 12-bp differences) with these sequences. One exception was the *amoA* gene of *Nitrosococcus oceanus*, which exhibited only a 2-bp difference. However, the *amoA* gene of this organism is more closely related to the *pmoA* genes of methanotrophs than to the *amoA* genes of nitrifiers so the high level of identity is not surprising (16).

Construction of clone banks and restriction fragment length polymorphism (RFLP) analyses. The size and purity of each PCR product were checked on 1% agarose gels (32). The PCR products were purified with a Qiagen PCR purification kit (Qiagen, Inc.) and were ligated into the pCR2.1 vector supplied with a TA cloning kit (Invitrogen) by following the manufacturer's instructions. Individual colonies containing inserts were suspended in 50 μl of water and boiled for 5 min, the cell debris was spun down, and 1-μl portions of the supernatant were used in PCR mixtures to reamplify the insert from the vector with the appropriate primers. The reamplified product was used in restriction digests along with tetrameric restriction enzymes. The 16S rRNA genes were digested with the enzymes *Msp*I, *Hha*I, and *Hae*III. The *pmoA* genes were digested with *Hha*I and a combination of *Msp*I and *Hae*III. Digests were resolved on 3% NuSieve GTG agarose (FMC) gels and were grouped manually based on the restriction patterns.

16S rRNA and *pmoA* genes from pure cultures. Pure cultures requiring methane for growth were obtained from enrichment cultures by using Lake Washington sediments (1b). Chromosomal DNA was isolated from each strain by using cells grown on agarose plates. Cells were washed from the agarose surface with 500 μl of TEN (50 M Tris EDTA, 150 mM NaCl), and the liquid was collected in 1.5-ml tubes. The tubes were centrifuged for 5 min at 14,000 rpm, and the supernatant was poured off. Each pellet was resuspended by adding 500 μl of TEN supplemented with 4 mg of lysozyme per ml and was incubated at 37°C for 1 h. Next, 50 μl of 20% sodium dodecyl sulfate was added to each tube, and the tubes were incubated in a 45 to 50°C water bath for approximately 30 min. DNA was extracted with phenol and was precipitated by using ethanol and standard procedures (32). DNA from each of the isolates was used in PCR mixtures as described above. The 16S rRNA genes were amplified by using bacterium-specific primers f27 and 1492r (13). The *pmoA* genes from each of the isolates were amplified by using primers A189gc and mb661 as described above.

Data analyses. Analyses and translation of DNA and DNA-derived polypeptide sequences were carried out by using Genetics Computer Group programs (Genetics Computer Group, Madison, Wis.).

Phylogenetic analysis. 16S rRNA gene sequences were compared with sequences in the small-subunit rRNA database of the Ribosomal Database Project (RDP) by using the Similarity_Rank program (22). 16S rRNA sequences were aligned manually with representative sequences of the nearest phylogenetic neighbors, as defined by the RDP, by using the SeqApp program. Dendrograms were constructed by using the programs DNADIST, DNAPARS, DNAML, NEIGHBOR, and SEQBOOT from the PHYLIP version 3.5c package (12). Tree files generated by PHYLIP were analyzed by using the program TreeView (29). The RDP program Check_Chimera was used to examine 16S rRNA gene sequences for chimeras. *pmoA* sequences were aligned manually with *pmoA* and *amoA* sequences obtained from the GenBank database. Dendrograms were constructed by using the programs PROTDIST, PROTPARS, NEIGHBOR, and SEQBOOT from PHYLIP, version 3.5c (12), and tree files were analyzed by using TreeView (29).

DNA sequencing. DNA sequencing of the 16S rRNA and *pmoA* genes was carried out with both strands by using an ABI Prism BigDye terminator sequencing kit (Applied Biosystems). The sequences were analyzed by workers at the University of Washington Center for AIDS Research DNA Sequencing Facility and the Department of Biochemistry Sequencing Facility, who used an Applied Biosystems automated sequencer.

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences determined in this study are AF150757 to AF150807.

RESULTS

RFLP analysis of known methanotrophs. Tetrameric restriction enzymes have been shown to be useful tools for screening

TABLE 2. Sizes of restriction fragments obtained from PCR-amplified products of methanotroph 16S rRNA grouped by genus

Genus[a]	Sizes of restriction fragments (bp)[b]		
	*Hha*I	*Msp*I	*Hae*III
Methylomicrobium spp.			
Methylomicrobium album (X72777)[c]	75, 126, 160, 170, 478	11, 33, **67**, 109, **347**, 442	34, 53, 59, 66, 66, 85, 99, 129, 418
Methylomicrobium agile (X72767)[c]	76, 124, 126, 160, 170, 352	11, 33, **67**, 109, **347**, 441	34, 52, 66, 67, 85, 99, 128, 153, 324
Environmental clone pAMC421	76, 126, 126, 160, 171, 350	**67**, 110, **348**, 484	34, 53, 66, 67, 99, 129, 561
Environmental clone pAMC466	76, 126, 126, 160, 171, 352	**67**, 110, **348**, 486	34, 53, 66, 67, 85, 99, 129, 478
Methylobacter spp.			
Methylobacter whittenburyi (X72773)[c]	75, 126, 126, 160, 170, 353	11, 33, 110, **414**, 442	34, 53, 66, 66, 85, 99, 129, 478
Methylobacter luteus (X72772)[c]	75, 126, 160, 170, 479	11, 33, 110, **414**, 442	34, 53, 66, 66, 85, 99, 129, 478
Isolate LW1	75, 126, 160, 170, 478	11, 32, 110, **414**, 442	34, 53, 59, 66, 85, 98, 195, 419
Environmental clone pAMC405	76, 126, 160, 171, 477	32, 110, **415**, 453	34, 59, 67, 99, 119, 129, 129, 374
Environmental clone pAMC415	76, 126, 160, 171, 478	11, 32, 110, **415**, 443	34, 53, 59, 66, 67, 85, 99, 129, 419
Environmental clone pAMC417	76, 126, 160, 172, 476	11, 32, 110, **415**, 442	34, 53, 59, 66, 67, 100, 129, 502
Environmental clone pAMC419	76, 126, 160, 171, 478	32, 110, **415**, 454	34, 66, 67, 85, 99, 182, 478
Methylomonas spp.			
Methylomonas methanica S1 (AF150806)[d]	76, 126, 644	11, 32, 360, 443	53, 66, 67, 85, 129, **446**
Methylomonas rubra (AF150807)[d]	26, 76, 126, 618	11, 32, 110, 250, 443	53, 66, 67, 85, 129, **446**
Isolate LW13	76, 126, 644	32, 360, 454	53, 66, 67, 85, 129, **446**
Isolate LW15	76, 125, 644	11, 32, 360, 442	66, 67, 85, 181, **446**
Isolate LW16	76, 126, 644	11, 32, 360, 443	53, 66, 67, 85, 129, **446**
Isolates LW19 and LW21	76, 126, 644	11, 32, 110, 250, 443	66, 67, 85, 182, **446**
Environmental clone pAMC434	76, 126, 644	11, 32, 360, 443	66, 67, 85, 182, **446**
Environmental clone pAMC435	76, 126, 316, 329	11, 32, 361, 443	66, 67, 85, 182, **446**
Environmental clone pAMC462	76, 126, 644	11, 32, 110, 250, 443	67, 182, 151, **446**
Methylosinus spp.			
Methylosinus trichosporium OB3b (AF150804)[d]	37, 62, 115, 172, **278**, 280	8, 86, 151, **155**, 255, 289	80, 85, 100, 186, 193, 300
Methylosinus sp. strain LAC (M95664)[c]	10, 37, 62, 115, 171, 267, **280**	86, 151, **155**, 263, 287	80, 85, 100, 186, 192, 299
Isolate PW1	37, 115, 172, **280**, 340	8, 86, 151, **155**, 255, 289	34, 37, 66, 80, 85, 156, 186, 300
Isolate LW2	37, 115, 170, **280**, 338	8, 86, 149, **155**, 255, 287	37, 80, 84, 100, 154, 186, 299
Isolates LW3, LW4, and LW8	37, 115, 171, **280**, 338	8, 86, 149, **155**, 255, 288	34, 37, 66, 80, 85, 154, 186, 299
Environmental clone pAMC447	37, 115, 172, **280**, 340	8, 86, 151, **155**, 255, 289	34, 37, 66, 80, 85, 156, 186, 300
Environmental clone pAMC451	37, 62, 115, 172, **278**, 280	8, 86, 151, **155**, 255, 289	80, 85, 100, 186, 193, 300
Environmental clone pAMC459	37, 62, 115, 172, **278**, 280	8, 151, **155**, 289, 341	80, 85, 100, 186, 193, 300
Methylocystis spp.			
Methylocystis sp. strain M (U81595)[c]	37, 112, 114, 172, **226**, 279	8, 149, 289, 494	80, 85, 100, 184, 191, 300
Methylocystis parvus OBBP (AF150805)[d]	37, 112, 115, 172, **226**, 289	8, 86, 149, 155, 255, 289	37, 80, 85, 100, 154, 186, 300
Isolate LW5	37, 112, 115, 172, **228**, 280	8, 86, 151, 155, 255, 289	37, 80, 85, 100, 156, 186, 300
Methylococcus spp.			
Methylococcus sp. strain Texas (X72770)[c]	2, 75, 126, 160, 165, **197**, 278	177, 340, **486**	34, 35, 59, 66, 151, 182, 476
Methylococcus sp. strain Bath (X72771)[c]	2, 76, 126, 160, 165, **197**, 279	177, 341, **487**	34, 35, 59, 67, 151, 182, 477
Methylocaldum spp.			
Methylocaldum tepidum (U89297)[c,e]	2, 160, 163, 197, **208**, 280	8, 70, 177, **340**, 415	34, 68, 92, 151, 187, 478
Methylocaldum szegediense (U89300)[c,e]	2, 160, 164, 197, **208**, 279	8, 66, 70, 176, **341**, 349	34, 53, 92, 151, 202, 478

[a] As determined by a phylogenetic analysis of sequences.
[b] Boldface type indicates OTUs for the genera determined by using only the portion of the 16S rRNA that would be PCR amplified with the primers used in this study.
[c] The data in parentheses are GenBank nucleotide sequence accession numbers. Patterns were predicted by using sequences deposited in the GenBank database.
[d] Data obtained in this study.
[e] Restriction fragments for the first 1,010 bp of 16S rRNA.

environmental clone libraries by RFLP analysis (10, 21, 27, 30, 34, 36). Common restriction fragments obtained from such analyses that distinguish between taxonomic groups are known as operational taxonomic units (OTUs) (27). Identification of OTUs for methanotrophs would facilitate rapid screening of both isolates and environmental clones. Therefore, a number of representative methanotrophic 16S rRNA genes available from the GenBank database were examined by performing computer-aided digestion with the tetrameric restriction enzymes *Msp*I, *Hha*I, and *Hae*III to determine whether OTUs could be identified. We predicted that these enzymes would produce useful patterns for regions used previously for PCR analysis (17), and a comparative computer analysis revealed that each genus could be identified by a distinct set of patterns (Table 2). To test our predictions experimentally, the same PCR products were generated by using DNA from representative strains and these PCR products were digested by the three restriction enzymes. Most of the RFLP patterns obtained for the strains tested corresponded to the patterns predicted on the basis of the previously described sequences; exceptions were the *Methylomonas methanica* S1, *Methylomonas rubra*, *Methylocystis parvus* OBBP, and *Methylosinus trichosporium* OB3b patterns. The discrepancies observed suggested that there may have been errors in the sequences deposited previ-

ously. The 16S rRNA genes from these cultures were resequenced, and significant apparent errors were identified in the original sequences. The new sequences which we obtained were 87 to 99% identical to the previously described sequences and matched the RFLP patterns obtained for the digests with chromosomal DNA, suggesting that the new sequences are correct. The RFLP patterns of the new 16S rRNA gene sequences also clearly fit into the OTUs defined for the respective genera (Table 2). The corrected sequences were especially significant for the type II *Methylosinus* and *Methylocystis* strains as only 10 16S rRNA gene sequences have been described for type II methanotrophs. It should be noted that many of the remaining eight *Methylosinus* and *Methylocystis* 16S rRNA gene sequences in the database do not produce the correct OTUs when they are analyzed in silico and may contain sequence errors in addition to ambiguous bases. All of the reference sequences used in our analyses contained genus-specific OTUs, and we were careful to choose the most accurate and complete sequence when possible.

In most cases, the RFLP patterns observed with *Msp*I digests were sufficient to differentiate between methanotroph genera. The genus *Methylomonas* was the only genus whose members exhibited a clearly distinct OTU in *Hae*III-digested sequences. In addition, the enzyme *Hha*I produced patterns that were useful for differentiating between the type II methanotrophic genera, *Methylosinus* and *Methylocystis*. Within each genus, the patterns obtained for *Msp*I- and *Hha*I-digested sequences were often very similar. In these cases the patterns observed with *Hae*III digests were used to differentiate between different clones and pure cultures. The sequences in Table 2 were analyzed by using only those bases that would be amplified with the genus-specific primers used in this study. The nonmethanotrophic representatives of the α- and γ-*Proteobacteria* tested did not exhibit any methanotrophic OTUs when they were digested in silico (data not shown).

pmoA PCR products were also analyzed both in silico and experimentally with *Msp*I, *Hae*III, and *Hha*I. Although these enzymes were useful for distinguishing between *pmoA* genes from different strains, no genus-specific OTUs could be identified.

Characterization of 16S rRNA and *pmoA* genes in new Lake Washington methanotrophic isolates. Twelve pure cultures that required methane for growth were obtained from enrichment cultures established with Lake Washington sediment (33a). Sequencing of the 16S rRNA genes of these isolates revealed one *Methylobacter* strain, five *Methylomonas* strains, one *Methylocystis* strain, and five *Methylosinus* strains. The OTUs predicted for the 12 Lake Washington strains (LW and PW strains) corresponded to the expected genera (Table 2). The *pmoA* genes of these isolates were also sequenced and screened by performing RFLP analyses. The results of an analysis of the *pmoA* sequences in the database in addition to our new *pmoA* sequences were used to design a primer specific for *pmoA* that should not amplify *amoA* (see above). The new *pmoA* primer, mb661 (Table 1), was tested with more than 10 *amoA* sequences available in the GenBank database and exhibited low levels of identity (9 to 12 mismatches) with these sequences. No product was obtained in PCRs in which *Nitrosomonas europaea* DNA was used.

Characterization of 16S rRNA and *pmoA* genes in natural methanotroph populations. (i) 16S rRNA gene sequences. 16S rRNA PCR products obtained by using target DNA extracted from Lake Washington sediment samples were used to construct gene libraries. The primers used to construct these libraries were the methanotroph phylogenetic group-specific primers described above and shown in Table 1 (17). A total of 200 randomly selected clones containing inserts were subjected to RFLP analyses and placed into groups based on their representative RFLP patterns. The 200 clones fell into 38 groups, only 15 of which contained more than one clone. All 38 groups were examined to determine whether any of the defined methanotrophic OTUs were present (Table 2). Based on this parameter, six groups were found to be groups that contained methanotrophic sequences. Clones representing each of these six groups were used for sequencing, and the data suggested that they were methanotroph 16S rRNA genes based on a comparison with other 16S rRNA genes. Ten clones that did not contain the defined methanotrophic OTUs were also used for partial sequencing. None of the additional 10 sequences were methanotrophic 16S rRNA gene sequences based on a comparison with other sequences in the RDP, which supported the validity of the OTU analysis.

TABLE 3. Grouping of 16S rDNA environmental clones from Lake Washington sediment

16S rRNA environmental clone	RDP similarity rank		Pure-culture representative
	Organism	Value	
pAMC405	*Methylobacter luteus*	0.861	None
pAMC415	*Methylobacter luteus*	0.875	LW1[a]
pAMC417	*Methylobacter luteus*	0.869	None
pAMC419	*Methylobacter whittenburyi*	0.714	None
pAMC421	*Methylomicrobium agile*	0.788	None
pAMC466	*Methylomicrobium agile*	0.782	None
pAMC434	*Methylomonas methanica*	0.925	LW15
pAMC435	*Methylomonas methanica*	0.925	None
pAMC462	*Methylomonas methanica*	0.830	None
pAMC447	*Methylosinus* sp. strain B-3060	0.825	None
pAMC451	*Methylosinus* sp. strain B-3060	0.841	None
pAMC459	*Methylosinus* sp. strain B-3060	0.847	None

[a] Sequences differ at two nucleotides.

The 16S rRNA gene sequences of the six methanotroph clones included four *Methylobacter* sequences (pAMC405, pAMC415, pAMC417, and pAMC419) and two *Methylomicrobium* sequences (pAMC421 and pAMC466) (Table 3). No sequences were obtained for the remaining six genera. However, representatives of the genera *Methylomonas*, *Methylosinus*, and *Methylocystis* were obtained as pure cultures that were isolated from the same sediment. Based on our sequence data for these isolates, we designed new primers to specifically amplify *Methylomonas* sequences and *Methylosinus* and *Methylocystis* sequences (Mm835 and Type2b, respectively) (Table 1). Additional gene libraries were constructed by using these primers. For each library, 50 clones were used in RFLP and OTU analyses. For the *Methylosinus-Methylocystis* library, six groups were obtained, and three of these had *Methylosinus*-type OTUs (pAMC447, pAMC451, and pAMC459) (Table 3). The 50 clones in the *Methylomonas* gene library fell into five groups, and three of these had the correct OTUs (pAMC434, pAMC435, and pAMC462) (Table 3). The six clones in the *Methylosinus* and *Methylomonas* gene libraries were sequenced. For each of these libraries, the clones that did not contain the appropriate OTUs were partially sequenced. None of the clones without the appropriate OTUs contained methanotrophic 16S rRNA genes. Our analysis of the environmental clones is summarized in Table 3. An environmental clone (pAMC434) identical to a Lake Washington isolate was obtained for one *Methylomonas* strain, and a clone (pAMC415)

TABLE 4. Levels of identity for the *pmoA* products of environmental clones and pure cultures of methanotrophs

Clone or culture	Genus	% Identity[a]																										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
1. pAMC503	*Methylobacter*		97	95.9	93.5	98.2	78	79.3	85.8	88.2	88.2	87.6	87.6	89.3	89.3	66.3	69.2	68.6	66.9	66.9	88.8	88.8	88.2	87.6	87.1	78.5	63	51.6
2. pAMC511	*Methylobacter*	93.3		95.3	96.4	95.3	78.1	79.9	83.4	85.8	85.8	85.2	85.2	87	87	65.1	68	67.5	65.7	65.7	86.4	86.4	86.4	85.2	84.7	79.1	61.7	52.2
3. pAMC523	*Methylobacter*	87.8	87.4		93.5	95.3	79.3	79.3	84	86.4	86.4	85.8	85.8	87.6	87.6	65.7	68.6	68	66.3	66.3	87	87	86.4	85.8	85.3	80.4	63	52.9
4. pAMC524	*Methylobacter*	87.6	94.3	86.8		91.7	78.7	78.1	84.6	87	87	86.4	86.4	85.8	85.8	66.9	69.2	68.6	67.5	67.5	85.2	85.2	85.2	86.4	85.9	79.1	63.6	51.6
5. pAMC528	*Methylobacter*	94.7	90.6	88.2	87.8		76.3	78.1	85.2	87.6	87.6	87	87	89.3	89.3	65.1	68	67.5	65.7	65.7	88.8	88.8	88.2	87	86.5	77.3	61.7	52.9
6. pAMC501	*Methylococcus*	70.9	69.9	70.7	70.1	69.7		92.3	74	74.6	74.6	75.1	75.1	75.1	75.1	67.5	65.1	64.5	68	68	74.6	74.6	74.6	75.1	73.6	92	63.6	54.1
7. pAMC512	*Methylococcus*	68.5	70.7	68.7	69.5	68.9	78.2		73.4	74	74	74.6	74.6	74	74	66.3	65.1	64.5	66.9	66.9	73.4	73.4	73.4	74.6	73.6	92.6	61.7	56.1
8. pAMC507s	*Methylomicrobium*	79.5	79.5	76.8	78.5	79.1	72.4	69.7		97.6	97.6	98.2	98.2	84.6	84.6	65.1	66.9	66.3	64.5	65.7	84.6	85.2	84	98.2	97.5	74.2	61.7	49.7
9. pAMC509	*Methylomicrobium*	79.7	80.1	77.4	79.5	79.3	72	69.7	98.8		100	99.4	99.4	85.8	85.8	66.9	68.6	68	67.5	67.5	85.8	86.4	85.2	99.4	98.8	74.8	63.6	51.6
10. pAMC519	*Methylomicrobium*	79.5	79.7	77.2	79.1	78.9	72	69.7	98.4	99.6		99.4	99.4	85.8	85.8	66.9	68.6	68	67.5	67.5	85.8	86.4	85.2	99.4	98.8	74.8	63.6	51.6
11. pAMC521	*Methylomicrobium*	79.7	79.7	77.4	79.1	79.3	72.4	70.3	99	99.4	99		100	85.8	85.8	66.9	68.6	68	67.5	67.5	85.8	86.4	85.2	100	99.4	75.5	63.6	51.6
12. pAMC526	*Methylomicrobium*	79.9	80.3	77.2	79.3	79.5	72.4	69.7	99.2	99.6	99.2	99.4		85.8	85.8	66.9	68.6	68	67.5	67.5	85.8	86.4	85.2	100	99.4	75.5	63.6	51.6
13. pAMC507	*Methylomonas*	82.5	80.3	79.9	79.1	81.9	71	67.9	77.6	78.1	78.1	78.1	78		100	63.9	65.1	64.5	64.5	64.5	99.4	98.2	97.6	85.8	85.3	74.2	60.5	51
14. pAMC514	*Methylomonas*	82.7	80.5	80.1	79.3	82.5	71.4	67.9	78	78.1	78.1	78.1	78.3	99.4		63.9	65.1	64.5	64.5	64.5	99.4	98.2	97.6	85.8	85.3	74.2	60.5	51
15. pAMC510	*Methylosinus*	63.5	63.4	63.9	64.4	64.2	71.2	67.6	66.3	66.9	67.3	67.3	66.7	64.8	64.8		91.7	92.3	98.8	98.8	63.9	64.5	63.9	66.9	65.4	67.9	90.8	48.1
16. LW2	*Methylocystis*	64.5	64.1	65.2	64	65	67.4	68.4	65.9	66.3	66.7	66.9	66.3	65.5	65.5	88.2		98.2	91.7	91.7	65.1	65.7	65.1	68.6	67.9	65.4	87.1	47.5
17. LW5	*Methylocystis*	64.2	63.3	64.5	63.1	64.1	68.1	69.2	65	65.4	65.8	65.9	65.4	65.4	65.4	88	93.9		92.3	92.3	64.5	65.1	64.5	68	67.3	65.7	89	48.2
18. PW1	*Methylosinus*	63.7	63.3	64.3	64.1	63.9	71.4	67	66.5	67.1	67.5	67.5	66.9	64.4	64.4	97.8	87.8	88.2		98.8	64.5	65.1	64.5	67.5	66	68.5	90.8	48.7
19. LW3	*Methylosinus*	63.4	63	64	63.8	63.6	71.5	66.9	67.3	67.9	68.3	68.3	67.7	64.9	64.9	97.4	87.8	88	98.4		64.5	65.1	64.5	67.5	66	68.5	90.8	48.7
20. LW21	*Methylomonas*	83.3	82.1	80.9	81.3	82.7	71.4	67.1	78.1	78.3	78	78.3	78.5	92.3	92.9	63.4	64.3	64	63.9	63.7		98.2	97.6	85.8	85.3	73.6	60.5	51
21. LW13, LW16, LW19	*Methylomonas*	82.5	81.5	79.7	80.5	82.1	70.1	67.3	78	78.1	78.1	78.1	78.3	90	90.6	63.5	63.7	63.4	63.3	63.1	94.5		98.2	86.4	85.9	73.6	61.1	52.2
22. LW15	*Methylomonas*	83.5	82.5	80.5	81.3	82.7	71	67.9	77.8	78.7	78.3	78.3	78.5	90.9	91.1	63.6	64.3	64.4	63.3	63.4	95.1	93.9		85.2	84.7	73.6	60.5	51.6
23. LW1	*Methylomicrobium*	79.9	80.3	77.6	79.7	79.5	72.2	69.9	98.8	99.6	99.2	99.4	99.6	78.3	78.3	66.9	66.5	65.6	67.1	67.9	78.5	78.3	78.9		99.4	75.5	63.6	51.6
24. BG8	*Methylomicrobium*	79.5	79	75.8	78	78.4	71.2	68.5	98.8	99.2	98.4	98.6	99.2	77.6	76.9	65.4	65.2	63.9	65.8	66.4	77.1	76.7	77.1	98.8		75.2	62.8	51.6
25. Mc	*Methylococcus*	69.9	70.7	71.3	71.9	69.3	87	82.3	72.4	72.5	72.4	73	72.4	70.9	70.9	71.5	68.6	68.9	72	71.3	71.3	70.3	71.1	72.6	71.4		64	55.4
26. OB3b	*Methylosinus*	61	60.8	61.4	61	61.6	70.6	65.5	64	64	64.4	64.6	64.4	63.4	63.6	89.2	84.7	84.7	88.1	88.4	61.6	60.9	60.2	64.2	63.7	70.3		44.9
27. *N. europaea*	*Nitrosomonas*	60.2	59	57.8	57.8	60.3	60	62.6	59.6	60.2	60.3	60.2	60.2	60.9	60.6	60.7	59.6	58.1	61.8	60.7	61.3	60.6	62.4	60.4	58.8	62.6	57.1	

[a] The values on the upper right are levels of amino acid identity for translated *pmoA* products, and the values on the lower left are levels of nucleotide identity.

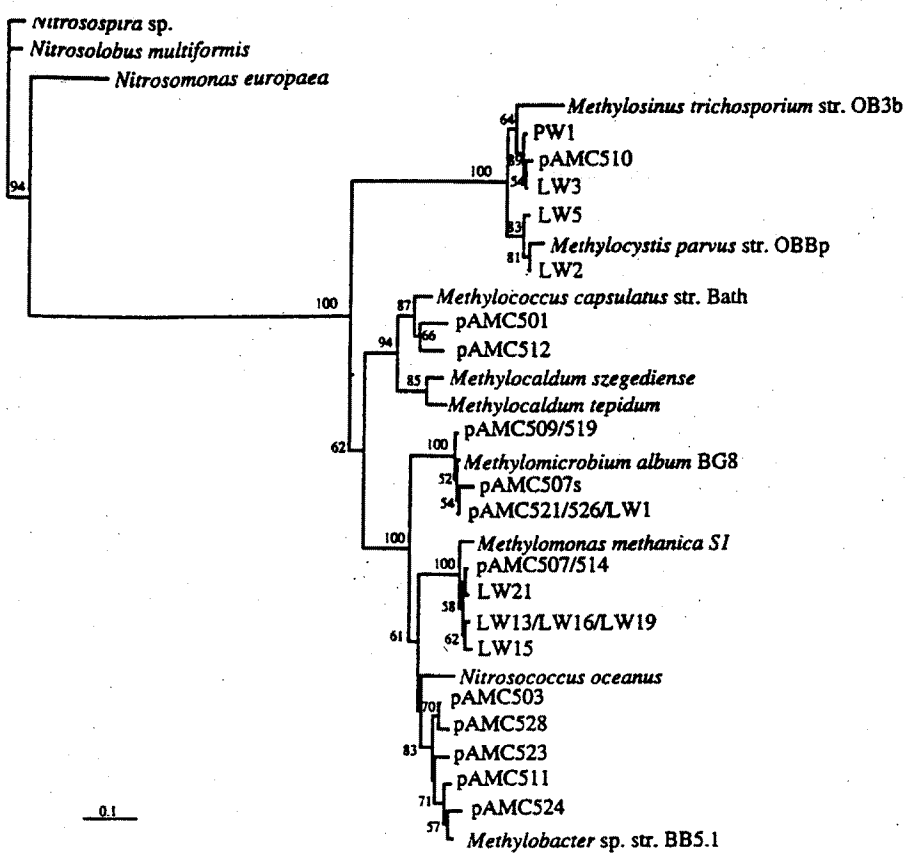

FIG. 1. Phylogenetic analysis of the derived amino acid sequences encoded by *pmoA* genes. Bootstrap values greater than 50% based on 100 replicates are shown near the branch points. The bar represents 10% sequence divergence as determined by measuring the lengths of the horizontal lines connecting two species.

that differed by only 2 nucleotides from a Lake Washington isolate was obtained for a *Methylobacter* strain. No other clones exhibited such close identity with any of the Lake Washington isolates.

(ii) *pmoA* sequences. The new *pmoA*-specific primers were used to amplify partial *pmoA* gene products from DNA extracted from Lake Washington sediment, and these PCR products were used to construct gene libraries. A total of 200 clones containing inserts were subjected to RFLP analysis with the tetrameric restriction enzymes *Msp*I plus *Hae*III and *Hha*I. The 200 clones fell into 34 groups, and only 8 of these groups contained more than one clone. Clones representing 24 of the groups were sequenced, and 15 of these clones were *pmoA* gene sequences. No *amoA* sequences were obtained. Pairwise comparisons of translated amino acid sequences for the *pmoA* PCR products obtained from environmental samples and from pure cultures indicated levels of identity ranging from 63.9 to 100% (Table 4). An examination of the nucleotide sequences from the same region revealed levels of identity ranging from 63 to 99.6% (Table 4). Analysis of this larger data set confirmed that it was not possible to identify OTUs for *pmoA* by using these RFLP profiles.

The 15 environmental *pmoA* sequences were compared to previously described *pmoA* sequences and were found to group with sequences from members of previously described genera (Fig. 1). These sequences included one *Methylosinus* sequence, two *Methylococcus* sequences, five *Methylomicrobium* sequences, two *Methylomonas* sequences, and five *Methylobacter* sequences. When these sequences were examined, we identified two clones that exhibited 100% amino acid identity with a type I methanotrophic isolate from Lake Washington (LW1). The amino acid sequences of some clones were identical, but the nucleotide sequences were different. In these cases, both clones are shown in Table 3. For all of the environmental clones and Lake Washington isolates, the *pmoA* gene obtained exhibited a higher level of identity with other *pmoA* genes than with a homologous gene, *amoA* from *Nitrosomonas europaea* (Table 4). The levels of nucleotide sequence identity with *amoA* ranged from 57.8 to 62.6%, while the levels of amino acid identity with the *amoA* product were 47.5 to 56.1%.

Phylogenetic analyses. The 16S rRNA and *pmoA* sequences obtained from pure cultures and environmental clones were subjected to phylogenetic analyses by using PHYLIP. In general, most of the new sequences grouped within the range of the previously described sequences (Fig. 1 through 3). However, one group of 16S rRNA sequences formed a distinct new cluster in the type II methanotrophs, which was supported by bootstrap values (Fig. 3). This group comprised isolates LW3 and PW1 and clone pAMC447. The diversity of both the 16S rRNA and *pmoA* representatives was much greater than the diversity found previously in peat or marine environments and spanned the known diversity of methanotrophs, except that we found no 16S rRNA sequences that represented the genera *Methylococcus*, *Methylosphaera*, and *Methylocaldum*. However, we identified two environmental *pmoA* clones that grouped with the genus *Methylococcus*, although no *Methylocaldum*- or *Methylosphaera*-like *pmoA* sequences were found.

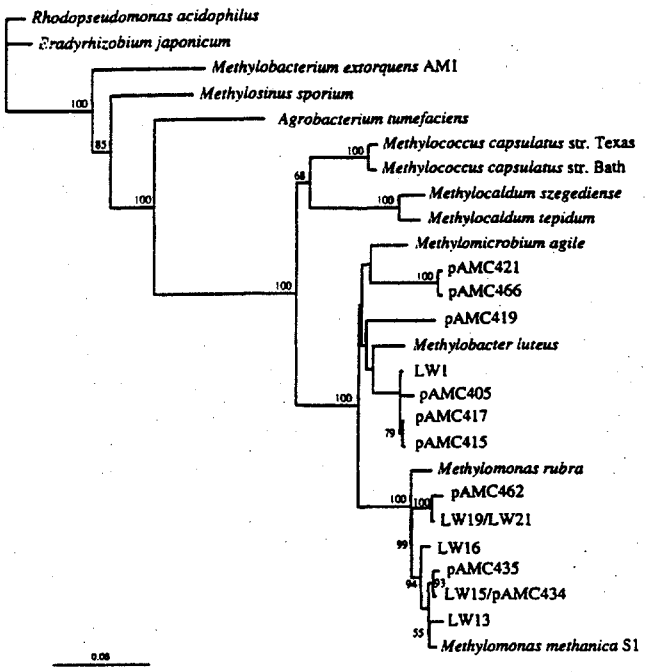

FIG. 2. Phylogenetic analysis of 16S rRNA genes from type I methanotrophs. Bootstrap values greater than 50% based on 100 replicates are shown near the branch points. The bar represents 5% sequence divergence as determined by measuring the lengths of the horizontal lines connecting two species.

DISCUSSION

Methanotrophic bacteria are important environmentally due to their role in carbon and oxygen cycling, as well as their use in bioremediation strategies. In order to more fully apply molecular techniques associated with these important bacteria, more information regarding the diversity of in situ populations in various environments is needed. Molecular tools are especially important because many methanotrophs are difficult to isolate on agar plates, which makes growth-based assessment of natural populations problematic (15). The ability to rapidly assess and monitor natural populations of methanotrophs by using molecular techniques holds great promise for understanding the complex role of these bacteria in nature.

Although there are currently primers for studying both 16S rRNA and *pmoA* genes of methanotrophs, these primers have some disadvantages for studying natural populations of the organisms. The 16S rRNA primers currently available were based on a relatively small sequence database. In addition, our study showed that some of the previously described sequences on which the primers were based contain errors that make accurate primer design difficult. In our study, these primers detected only a small subset of the existing methanotroph diversity in Lake Washington samples, and there was specific underrepresentation of the type I *Methylomonas* strains and all of the type II strains (both *Methylosinus* and *Methylocystis* strains). The previously described type I primers, Mb1007r and Mc1005r (17), were found to be sufficient for detecting these groups of methanotrophs. The *pmoA* primers that are available have a disadvantage opposite that of the 16S rRNA primers in that they amplify both *amoA* and *pmoA*, which makes them too unspecific for methanotroph-specific studies. Based on the sequences generated in this study, we designed new primers for methanotroph 16S rRNA and *pmoA* genes that appear to be more useful for studying methanotroph diversity in freshwater environments.

Using the newly developed primers (in addition to 16S rRNA primers Mb1007r and Mc1005r), we analyzed the 16S rRNA and *pmoA* genes in pure cultures isolated from Lake Washington and in environmental clone libraries obtained from the same sediment. We identified a broad diversity of both of these genes, including 13 new type I 16S rRNA genes, 7 new type II 16S rRNA genes, and 18 new *pmoA* genes, 5 of which grouped with *pmoA* sequences from type II strains. It is especially important to have additional type II gene data, as the database contains fewer type II sequences than type I sequences. However, it is equally important to have added environmental type I sequences to the database, as only two such sequences, both from marine environments, have been described. We did not detect any 16S ribosomal DNA (rDNA) sequences that grouped with the thermophilic methanotrophs belonging to the genera *Methylococcus*, *Methylocaldum*, and *Methylothermus*, nor did we detect any *Methylosphaera*-like sequences. Since Lake Washington sediment is a freshwater environment that stays at moderately low temperatures year-round (10 to 12°C), these results were not surprising.

So far, the phylogeny of the *pmoA* genes that have been described has mimicked the 16S rRNA phylogeny of the methanotrophs from which the *pmoA* genes were obtained. We observed the same correlation for the genes from new Lake Washington isolates described here. These combined results suggest that *pmoA* gene sequences may be useful in inferring 16S rRNA phylogeny of methanotrophs in situ (28). A comparison of the sequences from the environmental libraries of the methanotroph 16S rRNA and *pmoA* genes showed that the two types of sequences cover similar ranges of diversity, except that we did detect two *pmoA* sequences that are most similar to

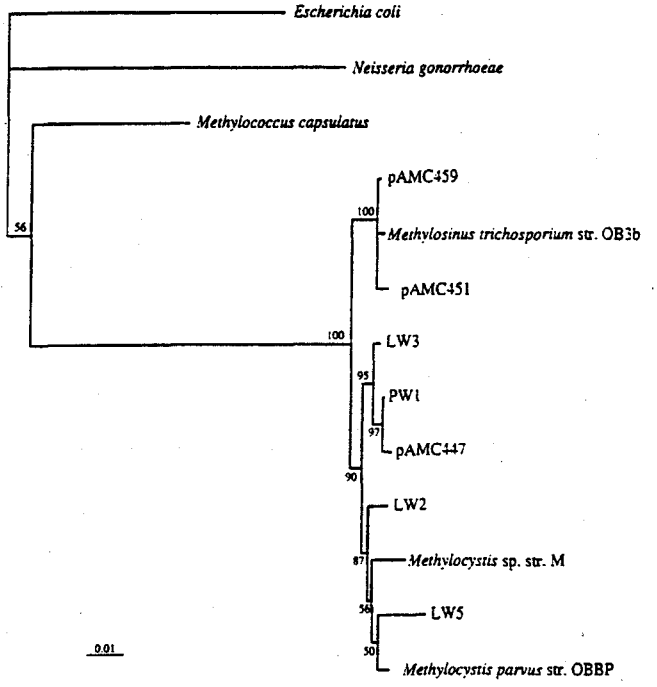

FIG. 3. Phylogenetic analysis of 16S rRNA genes from type II methanotrophs. Bootstrap values greater than 50% based on 100 replicates are shown near the branch points. The bar represents 1% sequence divergence as determined by measuring the lengths of the horizontal lines connecting two species.

Methylococcus pmoA, even though no *Methylococcus* 16S rDNA sequences were detected.

In addition to the new methanotroph primers, we also identified genus level OTUs for methanotrophs. Since all of the strains and sequences tested in this study exhibited complete correlation with the OTUs, it seems likely that these OTUs will be useful tools for screening methanotrophic isolates and environmental clone libraries from a wide range of environments. In addition, the OTUs can also be useful for screening enrichment cultures for the presence of nonmethanotrophs as an aid in facilitating isolation and purification of methanotrophs. Even though all of the methanotroph-specific primers used in this study showed no other close matches with any of the other organisms in the database, nonmethanotrophic sequences were obtained with all of the primers when environmental DNA templates were used. In this study, many of the nonmethanotrophic 16S rRNA sequences obtained were chimeric. As yet, no reliable protocol to circumvent these problems is in use. However, in the case of the methanotrophs, our data suggest that the OTUs defined in this study can be used as initial screening tools to distinguish between methanotroph and nonmethanotroph sequences in 16S rRNA gene libraries constructed from environmental samples.

The use of the new tools, new sequences, primers, and OTUs developed in this study demonstrated that the methanotrophs in Lake Washington sediment samples that could be detected by the methods which we used exhibit diversity as broad as the diversity of the known methanotrophs from all mesophilic environments. These results contrast with the results of studies of peat environments, which appear to contain only a limited group of type II strains (23, 25), and marine environments, which appear to be dominated by a limited group of type I strains (17, 33). The genes from two of the Lake Washington strains isolated from enrichment cultures were also found in the environmental clone libraries, suggesting that these two strains may be significant in the in situ populations. This is especially true for strain LW1 since both a 16S rDNA sequence and a *pmoA* sequence that exhibited high levels of identity to the same genes in this strain were found in the clone libraries.

The types of analyses carried out in this study cannot provide information concerning the dominant groups of methanotrophs in situ due to the known problems associated with PCR-based approaches, including differential amplification, artifactual PCR products, and inhibition of PCR amplification by contaminants (35). However, we are now in a position to develop and test hybridization probes for assessing the relative importance of methanotroph subgroups and specific strains (such as strain LW1) in detectable methanotroph populations.

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MALDI-TOF Analysis of Polymerase Chain Reaction Products from Methanotrophic Bacteria

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Polymerase chain reaction (PCR) assays were designed to amplify 56- and 99-base regions of the *pmoA* gene from *Methylosinus trichosporium* OB3b and *Methylomicrobium albus* BG8, two species of methanotrophic bacteria that are of interest for monitoring bioremediation activity. The PCR product sizes are in a mass range that is accessible to analysis by MALDI-TOF mass spectrometry. A rapid purification procedure using commercially available reversed-phase cartridges was applied prior to MALDI-TOF analysis. A small aliquot (1.5%, 1.5 μL) from a single 100-μL PCR reaction was sufficient for reliable detection. No cross-amplification products were observed when primers designed for one bacterial species were used with genomic DNA of the other species. The methodology described here has potential to allow less expensive and faster characterization of the ability of microbial populations to destroy pollutants in groundwater and soil at contaminated industrial sites.

Bacterial detection methods based on the polymerase chain reaction (PCR[1]) have been developed for a large number of organisms and are proving useful for analyzing microbial communities in natural habitats.[2] One area of application for such technology is in situ bioremediation, which exploits the ability of bacteria or other organisms to convert pollutants in groundwater or soil to less toxic forms.[3] To develop bioremediation into a cost-effective and reliable remediation strategy, it would be useful to have a rapid screening tool available to estimate the biodegradative populations available at the site and to monitor such populations once a bioremediation protocol is underway.

Molecular biological methods involving PCR that interrogate the genetic material of bacteria offer a promising basis for such a rapid screening method. However, current methods are time-consuming and difficult to carry out on site, mainly due to the requirement for electrophoretic separation of PCR products as a prelude to identification. Identification is usually carried out by estimating the size of the PCR product or by hybridizing it to a specific probe.

Recent advances in ionization processes, especially matrix-assisted laser desorption/ionization (MALDI)[4] and electrospray (ES),[5] have allowed for mass spectrometric analysis of large biomolecules, including DNA oligomers.[6–8] These developments suggested to several groups the possibility of using mass spectrometry as an alternative to electrophoresis for measuring DNA size in applications such as dideoxy sequencing or PCR product analysis. One obvious potential advantage of mass spectrometry for measuring DNA size is speed. In addition, mass spectrometry has the potential for detecting DNA signature molecules much more accurately than electrophoresis because it measures the mass-to-charge ratio and not the migration rate through a gel. Mass spectrometric analysis also circumvents the stringent solution conditions required for hybridization assays. MALDI combined with time-of-flight (TOF) mass spectrometry has been demonstrated for detection of PCR products from cloning vectors,[9] human DNA,[10–13] and bacterial DNA.[14] Various strategies for sequencing oligonucleotides using MALDI-TOF have also been described.[15–20] Muddiman et al. described the use of ES Fourier transform ion cyclotron resonance mass spectrometry for detec-

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tion of PCR products from three bacterial species with excellent resolution and mass accuracy.[21]

Although DNA species containing more than 500 bases have been detected,[22,23] MALDI-TOF is presently best suited to the analysis of DNA molecules that contain fewer than 100 bases, mainly due to considerations of sensitivity and resolution. Experimental refinements such as sample preparation,[24] new matrixes,[25] incorporation of modified nucleotides,[9,26-28] and instrumentation advances such as delayed extraction[19,29-31] continue to extend the accessible mass range. For MALDI-TOF detection, the development of PCR products of sizes *less* than 100 bases is currently desirable.[10,12] In contrast, agarose gel electrophoresis is usually applied to DNA containing *more* than 100 bases; for this reason, PCR products are rarely designed with sizes of less than 100 bases. However, there is no fundamental reason that smaller PCR products cannot be generated. The lower end of the size range for PCR products is determined by the primer length and sequence-dependent effects such as melting temperature and secondary structure. Given these constraints, the optimum size for PCR products tailored for MALDI analysis is presently in the 50−100-base range.

In addition to oligonucleotide size, another important issue in the application of MALDI to analysis of DNA is the necessity of a rapid and efficient sample preparation procedure. PCR products offer a particular challenge because they contain relatively high concentrations of salts, buffers, nucleoside triphosphates, primers, and other components that interfere with either desorption or ionization of the analyte in MALDI. Alkali metal cations form adducts with the negatively charged phosphodiester backbone to give a mixture of DNA salt forms, each with a different m/z. These salt forms appear in the mass spectrum as resolved adduct peaks for modest salt concentrations and smaller oligonucleotides or as broad unresolved peaks for higher salt concentrations and larger oligonucleotides. Various methods have been described for removing these interfering components from PCR products prior to mass spectrometric analysis.[10,11,13-21] The results presented here were obtained using a 5-min technique based on a commercially available reversed-phase cartridge, along with addition of ammonium-form cation-exchange beads[24] to the final sample.

Methanotrophic bacteria are of interest for in situ bioremediation of halogenated solvents such as trichloroethylene, dichloroethylene, and vinyl chloride.[32-34] Molecular probes and PCR primers have been designed for these bacteria using both 16S rRNA-based sequences and sequences specific to an enzyme that is diagnostic for these bacteria, particulate methane monooxygenase (*pmoA* gene sequences).[35-37] We have used the known sequence data for *pmoA* genes to design primers to generate short PCR products diagnostic for the two major groups of methanotrophs, the type I and type II strains. These PCR products are 99 and 56 bases long, respectively. This paper describes the application of MALDI-TOF to analysis of these diagnostic PCR products generated from a representative of each group—*Methylosinus trichosporium*, a type II methanotroph, and *Methylomicrobium albus*, a type I methanotroph. We demonstrate a methodology that includes PCR amplification, rapid reversed-phase purification, and MALDI-TOF detection of each of these products, as well as lack of amplification in negative control experiments.

EXPERIMENTAL SECTION

Chromosomal DNA was isolated from methanotrophic cultures grown on agarose plates. Cells were washed from the agarose surface using 1.5 mL of TEN (50 mM Tris EDTA, pH 8.0; 150 mM NaCl), and the liquid was collected in 1.5-mL microcentrifuge tubes. Tubes were spun for 5 min at 10000*g*, and the supernatant was poured off. The pellet was resuspended by adding 500 μL of TEN containing 4 mg/mL lysozyme and incubated at 37 °C overnight. Next, 50 μL of 20% SDS was added to the tubes and incubated in a 45−50 °C water bath until the solution was clear, approximately 30 min. DNA was extracted using 500 μL of phenol and precipitated using ethanol by standard procedures.[38]

Primers for short PCR products were developed for use with the type I and type II methanotrophs, *Methylomicrobium albus* BG8 and *Methylosinus trichosporium* OB3b, respectively. *pmoA* sequences from a large number of different methanotrophs were aligned and compared using the sequence editor program SeqApp.[39] Regions that were specific for each type of methanotroph were examined in detail, and the PCR primers were designed using these areas. The primers were designed to amplify all *pmoA* genes from each type of methanotroph but not the analogous

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Table 1. Primers and Sequences of the Type I (99-Base) and Type II (56-Base) PCR Products

	Type I Primers
forward	5′-GGC TGA ITT ICA AGG TTA ICA C-3′
reverse	5′-GCA ACG TCI TTA CCG AAA GTT-3′
	Type II Primers
forward	5′-GAI TAI ATC CGC ATG GTI GAG-3′
reverse	5′-ACG ACG TCC TTA CCG AAG GTI CG-3′

Type I Sequence (MW 30 680, Complement MW 30 364)
5′-GGC TGA CTT GCA AGG TTA CCA CTA TGT AAG AAC CGG TAC TCC AGA ATA TAT CCG GAT GGT TGA AAA AGG TAC TCT GAG AAC TTT CGG TAA GGA CGT TGC-3′

Type II Sequence (MW 17 282, Complement MW 17 202)
5′-GAA TAT ATC CGC ATG GTC GAG CGC GGC ACC GTG CGC ACC TTC GGT AAG GAC GTC GT-3′

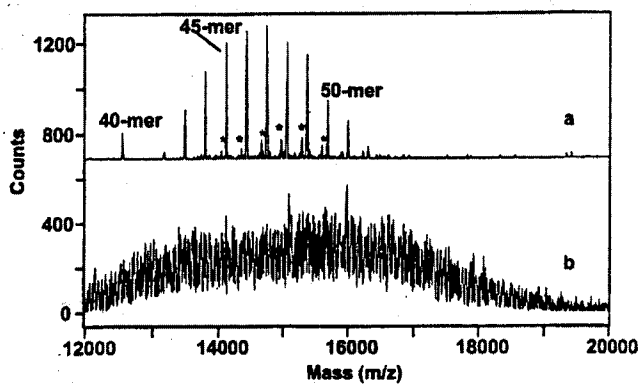

Figure 1. (a) Negative-ion MALDI-TOF mass spectrum of a pdA_{40}–pdA_{60} mixture, purified using the OPC procedure. Loss of HPO_3 is indicated by an asterisk. Accelerating voltage, −25 kV; grid voltage, 90.5%; guide wire voltage, 0.15%; extraction delay, 300 ns; low-mass gate, 10 000 Da. The grid voltage and guide wire voltages are expressed as a percentage of the accelerating voltage. (b) Same conditions as (a), but the pdA_{40}–pdA_{60} mixture was not purified.

subunit of the ammonia monooxygenase gene. Inosine bases were incorporated into the primers at some sites to increase their generality.[40]

Bacterial chromosomal DNA samples were amplified with these primers, yielding products 56 and 99 base pairs (bp) in length (Table 1). Amplification reaction mixtures contained 1 μL of native *Taq* polymerase (5 units/μL), 10 μL of GeneAmp 10× PCR buffer, 10 μL of a mixture that is 2 mM in each nucleoside triphosphate, 10 μL of a 10 μM solution of each primer, 2 μL of template DNA (100 ng/μL), and deionized water to bring the final volume to 100 μL. PCR reagents were obtained from Perkin-Elmer Applied Biosystems Division (Foster City, CA), and custom primers were synthesized and HPLC purified by Life Technologies (Gaithersburg, MD). Deionized water was obtained from a Millipore Corp. (Bedford, MA) system with Milli-RO and Milli-Q UV Plus modules. The PCR, carried out using a Perkin-Elmer 2400 thermal cycler, consisted of a 4-min hold at 94 °C; 10 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 45 s; 20 cycles of 90 °C for 30 s, 55 °C for 45 s, 72 °C for 45 s; followed by a 7-min extension at 72 °C. Initially, products were verified on a 1.5% agarose gel.

Purification of the samples was accomplished by using Oligonucleotide Purification Cartridges (OPC) from Perkin-Elmer Applied Biosystems using the manufacturer's desalting protocol, modified to elute the product in a smaller, 100-μL volume and to reduce wash solvent volumes as follows. The OPC column was conditioned with 1 mL of acetonitrile, followed by 1 mL of 2 M triethylammonium acetate (TEAA). The crude PCR reaction mixture was added to 1 mL of 0.1 M TEAA and introduced to the column, followed by washes with 1 mL of 0.1 M TEAA and 1 mL of deionized water. The PCR product was eluted in 100 μL of 1:1 water/acetonitrile. An equal volume of matrix solution (50 mg/mL 3-hydroxypicolinic acid (HPA) in 1:1 acetonitrile/water) was added to the purified sample, along with a small amount of Dowex 50W-X12 ion-exchange resin beads (Bio-Rad, Hercules, CA) in the ammonium form.[24] The HPA (98%, Fluka Chemical Corp., Ronkonkoma, NY) was purified by sublimation before use.

A 3-μL aliquot of the solution of PCR product and matrix, along with several ion-exchange beads, was transferred to the MALDI-TOF sample plate. The mixture was allowed to dry under ambient conditions, yielding a spot ~3 mm in diameter, and was subsequently analyzed in negative ion mode with delayed extraction using a Voyager DE MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA). The low-mass cutoff was applied to reduce detector saturation by matrix and other species with short flight times. Spectra shown represent the average of 20–150 laser shots, and other conditions for acquisition are listed with each figure.

External mass calibration of the 56-mer PCR products was accomplished using a mixture of synthetic polyadenylic acid oligomers, pdA_{40-60} (Pharmacia Biotech, Piscataway NJ), and verified with a synthetic 50-mer (Life Technologies) of sequence 5′-GGG TCT GAT CTT CTA CCC GGG CAA CTG GCC GAT CAT CGC GCC GCT GCA CG-3′ and a calculated molecular mass of 15 316 Da. A synthetic 99-mer (Life Technologies) of the type I sequence shown in Table 1 (30 680 Da) provided external mass calibration for the 99-mer PCR products.

RESULTS AND DISCUSSION

Figure 1a illustrates the performance of the OPC cleanup followed by delayed-extraction MALDI-TOF for a mixture of synthetic oligonucleotides in the size range targeted by our PCR strategy. This spectrum was obtained using a mixture of synthetic poly(adenylic acid) oligomers, pdA_{40-60}, with the delayed extraction conditions optimized for the 50-mer. In spectra obtained prior to OPC purification (Figure 1b), no distinct peaks were observed in a broad feature encompassing the m/z range of the sample. In the spectrum shown in Figure 1a that was obtained from the purified sample, distinct peaks, ranging from 10 to 12 Da in width, appear for the 40-mer through the 52-mer. In some cases, loss of HPO_3 (80 Da) is observed, presumably from the 5′ phosphate group. This has been attributed to solution-phase degradation or incomplete phosphorylation of the oligonucleotides.[24,41] At higher laser power, the dephosphorylated peaks became more prominent, indicating increased prompt fragmentation due to greater energy deposition in the sample. For these reasons, the use of 5′-phosphorylated oligonucleotides is, perhaps, not the best

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choice for calibration due to ambiguity in assigning m/z to various peaks, especially if the laser power is not carefully controlled. The accuracy of a three-point mass calibration obtained with the pdA_{40}, pdA_{45}, and pdA_{50} peaks was checked by applying it to the spectrum of a synthetic mixed-base 50-mer of calculated m/z 15 315 for the $[M - H]^-$ ion. The observed m/z for this 50-mer (see Figure 3, below) was 15 310, an error of 0.03%. The mass accuracy and resolution demonstrated in Figure 1a illustrate the utility of MALDI-TOF for analysis of a mixture of oligonucleotides that one might encounter in a multiplexed PCR, amplification of a polymorphic region, or detection of some single-base substitutions in suitably sized products.

As described above, PCR primers were designed to amplify 99- and 56-base regions respectively of *pmoA* from type I and type II methanotrophs. The PCR products were then purified and analyzed by MALDI-TOF. The reduced-volume OPC purification described in the Experimental Section required only an additional 5 min after PCR amplification. Depending on losses, the OPC purification yields a PCR product concentration comparable to or slightly less than that in the raw PCR mixture, because the volume in which the product is eluted from the column is the same as the PCR reaction volume (100 μL). Because this rapid method uses a solid sorbent, it is amenable to automation and parallel implementation. Other than addition of matrix and cation-exchange beads, no further manipulation or concentration of the products was necessary prior to MALDI analysis. We were able to observe spectra using a small aliquot (1.5 vol %) of a single OPC-purified 100-μL PCR mixture, although improved signal-to-noise ratios (S/N) could be obtained by concentrating the samples. Negative control experiments were performed by using type I primers with type II DNA and type II primers with type I DNA in the PCR. Blank reactions were performed by omitting bacterial DNA from the PCR mixture. No product was observed for either negative control experiment or the blank reaction.

Figure 2 shows MALDI mass spectra obtained from 1.5-μL aliquots taken from eight separate 100-μL PCRs using the type II primers. Three of these reactions contained chromosomal DNA from a type II methanotroph population, which the primers were designed to amplify. Of the remaining reactions, three contained chromosomal DNA from a type I methanotroph population, and two contained no added bacterial DNA. The PCR, OPC purification, and mass spectrometry were performed in a "blind" manner, in that the target DNA was added to the PCR reaction mixtures by one experimenter, with all subsequent steps performed by a second. The spectra obtained from the reactions containing the type II DNA clearly show peaks corresponding to the 56-mer product of the sequence given in Table 1, while the negative control (type I DNA) and blank reactions show no detectable 56-mer product. The 56-mer peaks in Figure 2 are somewhat broader than those observed in Figure 1, because the aim of the experiment leading to Figure 2 was determination of the presence of the PCR product rather than optimization of mass spectrometric resolution, which can be fairly time-consuming. Subsequent experiments (data not shown) have indicated that using the "autosampler" data acquisition mode of the Voyager DE instrument allows mass spectra of the type II PCR product to be obtained with consistently higher resolution. The results of Figure 2 demonstrate the sensitivity of MALDI-TOF for detection, the

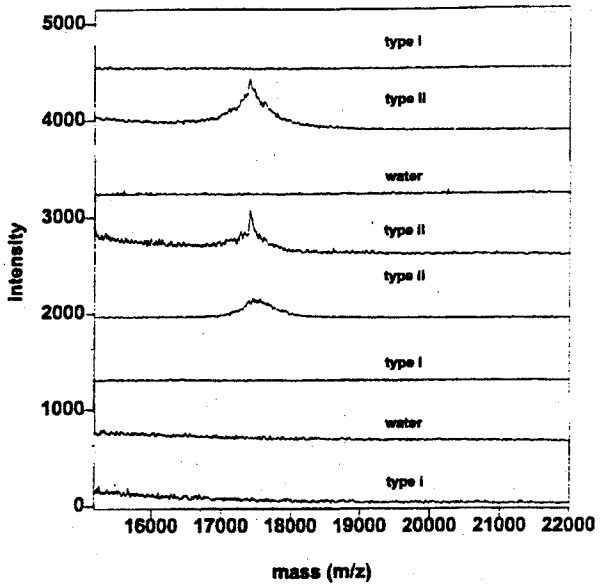

Figure 2. Negative-ion MALDI-TOF mass spectra obtained from PCR reactions using the type II primer set with either type I or type II chromosomal DNA or no added DNA ("water"). Accelerating voltage, −25 kV; grid voltage, 90.5%; guide wire voltage, 0.15%; extraction delay, 300 ns; low-mass gate, 10 000 Da.

specificity of the type II primer pair, the reproducible nature of the assay, and the lack of contamination.

While the 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, and the two strands could, conceivably, exhibit different MALDI detection efficiencies. Under conditions for which the two single strands are not resolved in the mass spectrum, the average m/z for the two strands is measured and is correlated with the number of bases in the product.[22] The two strands of the type II 56-base region targeted for amplification have calculated molecular masses of 17 282 and 17 202 Da, a difference of 80 Da, and should be resolved using conditions similar to those used for Figure 1a. These two species, however, are not the only ones that can be produced by the PCR. In the amplification product, each strand will have incorporated a primer with one or three inosine bases (see Table 1), giving slightly different molecular weights for the strands than quoted above. Furthermore, because the inosine bases allow nonspecific incorporation of nucleotides into the complementary strand during polymerase extension,[40] products can be generated with differing base compositions. Each product strand contains either one or three sites that can vary in base composition because they are paired with inosines in the complementary strand. The range in molecular mass imparted by this variation is approximately 120 Da, which is 3 times the mass difference contributed to an oligonucleotide by the largest (G) vs smallest (C) nucleotide units. With careful control of laser power and by searching for a favorable spot on the sample, sufficient resolution can be obtained to permit the observation of several of these species in the mass spectrum. As Figure 3 illustrates, while a single product dominates the spectrum, other species of similar size are also observed. The peak at m/z 17 320 falls within the range of predicted m/z values for the PCR product. The dominant peak at m/z 17 413 is slightly heavier than the predicted m/z range for either strand of the PCR product; the reason for this is not

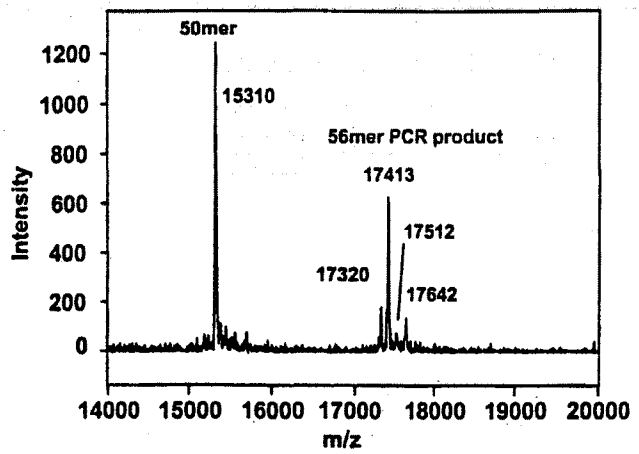

Figure 3. Negative-ion MALDI-TOF mass spectrum of 5 pmol of a synthetic 50-mer (m/z 15 315) and the type II PCR product. The spectrum was smoothed with a five-point Savitsky–Golay filter. Accelerating voltage, −25 kV; grid voltage, 90.5%; guide wire voltage, 0.15%; extraction delay, 300 ns; low-mass gate, 10 000 Da.

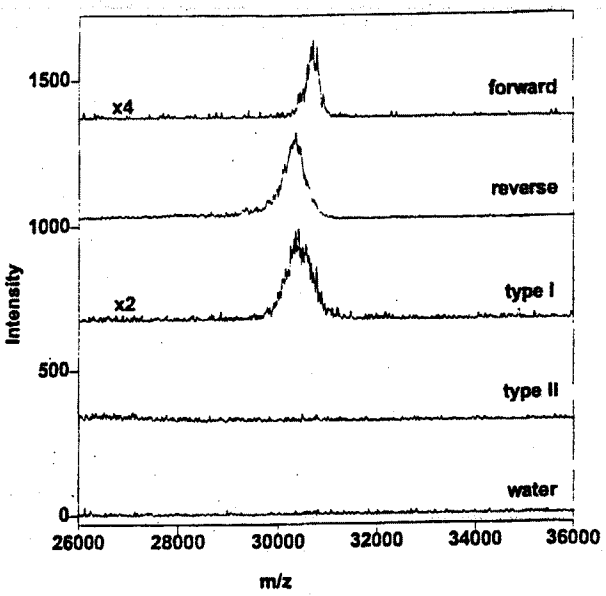

Figure 4. Negative-ion MALDI-TOF mass spectra obtained from (upper two spectra) synthetic 99-mers and (lower three spectra) PCR reactions using type I primers with either type I or type II chromosomal DNA or no added DNA. Approximately 100 pmol of each 99-mer was used for the upper two spectra, and the matrix for these two spectra was 50 mg/mL 3-HPA, 10 mg/mL picolinic acid, and 10 mg/mL ammonium citrate in 1:1 water/acetonitrile. The spectra were smoothed with a 19-point Savitsky–Golay filter. The "forward" 99-mer and the type I PCR product spectra were scaled by the factors indicated in the figure. Accelerating voltage, −25 kV; grid voltage, 92% (upper two spectra) or 77% (lower three spectra); guide wire voltage, 0.20% (upper two spectra) or 0.25% (lower three spectra); extraction delay, 400 ns; low-mass gate, 10 000 Da.

known, although the mass calibration was extrapolated into this region, possibly degrading the mass accuracy. The small peak at m/z 17 512 is also above the predicted m/z range for either product strand. Finally, the peak at m/z 17 642 could represent nontemplated enzymatic addition of an additional adenosine residue to the product observed at m/z 17 320. It is interesting that one product dominates the mass spectrum, due to either more efficient amplification or detection. Although one might initially attribute multiple peaks to mass spectral artifact peaks that can arise from sources such as prompt fragmentation of the PCR product or cation adduction, the lack of such features accompanying the synthetic 50-mer peak in Figure 3 indicates that these artifacts do not contribute significantly to the spectrum. The peaks observed in the region of the 56-mer are, therefore, most likely to be actual products of the PCR.

For larger PCR products, such as the 99-mer amplified from the type I methanotroph, the resolution of MALDI-TOF is currently not sufficient to resolve variations of the sort seen for the 56-mer, although these variations may contribute to the breadth of the observed peaks. The upper two spectra in Figure 4 are negative-ion MALDI-TOF mass spectra obtained from the single-stranded synthetic 99-mer corresponding to the sequence for the type I product given in Table 1 ("forward") and its complement ("reverse"), with calculated molecular masses that differ by 316 Da. These two spectra exhibit distinctly different centroids, although they are not well resolved from each other due to their breadth. The lower three spectra in Figure 4 represent the PCR products obtained using type I primers with either type I DNA, type II DNA (negative control), or no added DNA (blank). The expected 99-mer product is observed from the reaction containing the type I DNA, while no product is observed from the negative control or blank reactions. The peak obtained from MALDI-TOF analysis of amplified type I DNA is some 630 Da wide, or approximately two nucleotide units, slightly broader than the upper two spectra of the single-stranded 99-mers. The slightly broader peak width obtained from the PCR product may be due to the presence of the two strands of different mass, cation adduction, or fragmentation.

The mass spectra of PCR products shown in Figures 2 and 4 were all obtained from small aliquots (1.5 μL) of single 100-μL PCR mixtures. This is a significant improvement over our previous studies, in which several 100-μL PCR preparations were pooled and concentrated to yield sufficient product for MALDI detection.[10,14] The current level of sensitivity is sufficient for developing a practical screening procedure, as it reduces the reagent costs and time that would be required for performing and pooling multiple reactions and, in fact, uses quantities of PCR product similar to those typically used for more conventional hybridization or electrophoretic assays. The sensitivity level demonstrated here suggests that the PCR could be performed in smaller volumes, further decreasing reagent costs. The purification step could also be performed at reduced cost with smaller sorbent volumes, as the capacity of the OPC cartridges is significantly higher than the maximum yield of our PCR reactions. The cartridges have a specified capacity of 50 A_{260} units of DNA. If 100% efficient, our 100-μL PCR reactions would produce 100 pmol of double-stranded product (the amount of primer added.) For the 99-mer, this corresponds to 6 μg of DNA, or approximately 0.1 A_{260} unit, which is some 500-fold less than the capacity of the OPC cartridge. This type of purification could be adapted to a microtiter plate format and automated to allow high-throughput sample purification.

It is important to note that each stage of a procedure for a mass spectrometry-based analysis of microbial PCR products, including DNA isolation, PCR amplification, product purification, and MALDI-TOF analysis, must be reproducible and efficient to reliably detect product from a single reaction. We are currently

developing various controls to flag problems at different stages of the analysis.

CONCLUSIONS

We have demonstrated the design, amplification, purification, and analysis of short PCR products using an approach that exploits the speed and mass accuracy advantages of MALDI-TOF. This procedure shows promise as a high-throughput and accurate means of profiling the genetic potential of bacterial populations for methane-dependent cometabolism of small halogenated solvents. The design of short PCR products for other enzymatic degradation pathways relevant to bioremediation should also be feasible. As the mass range and other figures of merit for DNA analysis by MALDI-TOF (or other mass spectrometric techniques) continue to improve, the variety of information-bearing DNA assay products that can be "read out" by this method will also increase.

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ANALYSIS OF PCR PRODUCTS USING DELAYED-EXTRACTION MALDI-TOF

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Successful application of MALDI-TOF to analysis of nucleic acid samples requires a comprehensive approach to improving figures of merit such as mass range, resolution, and sensitivity. This approach must include both instrumental improvements and reliable and convenient methods for sample preparation. Alternatively, it is possible to design biological assays that produce information-bearing product molecules of smaller sizes currently amenable to analysis by MALDI.

Improvements in TOF instrumentation and sample preparation are beginning to allow the useful analysis of larger oligonucleotides. Delayed extraction (DE) applied to MALDI-TOF has allowed greatly improved resolution. Protocols for commercially-available oligonucleotide purification products can be modified slightly to produce MALDI-compatible samples.

Another perspective from which to approach mass spectrometry-based measurement methods is to design biological assays that yield smaller product molecules. For instance, products of the polymerase chain reaction (PCR) have generally been analyzed using electrophoretic techniques that are more suited to larger products (>100 bases), so PCR assays are typically designed to produce products in this size range. However, MALDI currently is better suited to oligonucleotides containing <100 bases. The information borne by a PCR product is either the presence or absence of the product (independent of size), or is encoded as variations in number or identity of only a few bases, with the remainder of the product serving an uninformative "carrier" role. Elimination of as much of the unnecessary, non-informative portion of the PCR product as practical can in favorable cases decrease the size of the product to a range amenable to analysis by MALDI.

We describe here the application of delayed-extraction MALDI-TOF to analysis of oligonucleotides in the size range of smaller PCR products. SIMION modeling of ion flight time distributions under delayed extraction conditions allows evaluation of the effects of practical hardware limitations that result in non-idealities such as fringing fields.

The mass spectrometer is an in-house constructed linear time-of-flight mass spectrometer with a 25.5 cm field-free region and electronics for delayed extraction experiments. The flight tube liner is floated at the potential of the multiplier. A pulsed nitrogen laser operating at 337 nm provides energy for desorption. Purified nucleic acid samples are mixed on a stainless steel probe tip with aqueous 0.3 M 3-hydroxypicolinic acid (HPA). A small number of dry cation exchange beads in the ammonium form are added to samples. Deionized water is used for all solutions to decrease salt content.

SIMION 3D, version 6.0,[1] was used to model the trajectories of ions under delayed-extraction conditions. Ions of various initial kinetic energies and directions appropriate to the MALDI process were examined. A user program allowed modeling of delayed extraction by changing the electrode potential of the sample probe. By varying initial ion velocities over the range 0-1500 m/s, a spread in calculated flight times results which gives an indication of mass spectral peak breadth. For accurate results, it was necessary to increase the SIMION trajectory quality to 22 (default value = 2). Doubling the density of the potential array produced no significant changes in the results. For an "ideal" TOF model in SIMION, electrodes and grids extend to the edges of the potential array, and no vacuum chamber ground can is included.

SIMION models of "real" TOF instruments include a grounded vacuum can and mounting plates for grids that mimic the hardware geometry of our instrument. When optimum extraction delay times are chosen the variation in flight time for ions with initial velocities ranging from 0 to 1500 m/s is smaller for the "perfect" TOF than for the "real" TOF. Using SIMION also allows modeling of off-axis initial ion velocities, which may broaden the observed spectral peaks by a factor of approximately 2-3.

Figure 1 is the DE-MALDI-TOF positive-ion spectrum of a mixture DNA homo-oligomers, $pd(A)_{40\text{-}60}$, purchased from Pharmacia. The width of the 45-mer peak (FWHM) is ~21 Da. The spectrum is the average of 20 laser shots and a 13-point moving-average smooth was applied. The total accelerating potential was 6.6 kV and the delay time was 4.29 µs. The sample was desalted by two spins (1x 0.1 M ammonium citrate, 1x deionized water) through a Rainin microcentrifuge-tube format 10,000 Da molecular weight cutoff filter. Approximately 10 pmol of each oligomer was applied to the probe tip.

Figure 2 is the DE-MALDI-TOF spectrum of yeast phenylalanine-specific transfer RNA (tRNA), MW 25817 Da. The width of the main, narrow peak at its base is ~100 Da, which is better than single-base resolution. The spectrum represents the average of 20 laser shots, and a 19-point moving-average smooth was applied. The total accelerating potential was 6.6 kV and the delay time was 6.45 µs. The sample was purified as described above for the $pd(A)_{40\text{-}60}$ sample. 50-100 pmol tRNA was applied to the probe tip.

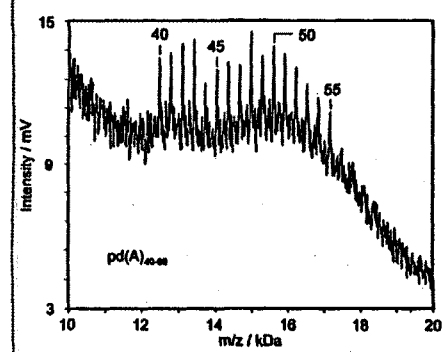

Figure 1

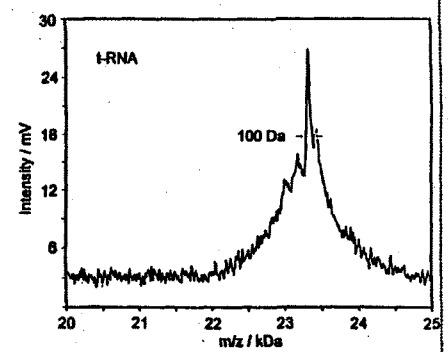

Figure 2

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96-WELL MICROTITER-FORMAT PURIFICATION OF DNA FOR MALDI-TOF ANALYSIS

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MALDI-TOF MS has the potential for fast, accurate, large-scale polymerase chain reaction (PCR) product screening. Analysis of DNA from sources such as solid-phase synthesis, enzymatic digestion, sequencing reactions and amplification reactions is plagued by the presence of salts and other impurities resulting in reduced sensitivity, degraded resolution or both in the mass spectrum. Currently, it is possible to obtain single base resolution of PCR products up to about 100 bases with purification to remove PCR components such as Tris-HCl, KCl, $MgCl_2$, gelatin, *Taq* DNA polymerase, and deoxynucleoside triphosphates. However, procedures to purify DNA products prior to analysis are time-consuming and labor intensive. We demonstrate an automatable, reverse-phase method to purify 96 DNA samples simultaneously using a microtiter-format. By developing an appropriate search pattern, we acquire sample data in autosampler mode which further reduces the need for human intervention.

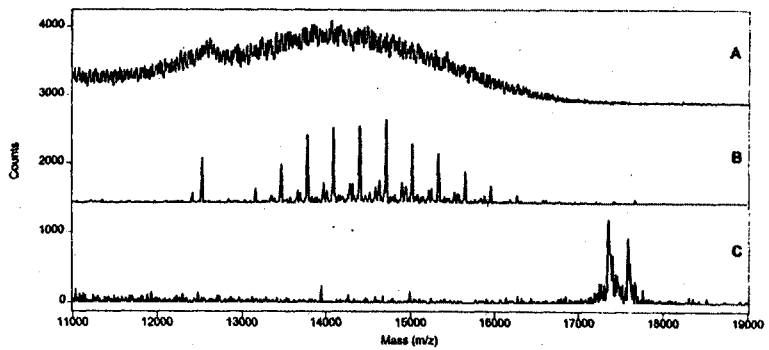
PCR product purification: A 56-base pair bacterial DNA sequence was amplified by PCR in forty-eight 100 µl reactions which were pooled in order to negate any slight differences in individual amplification reactions. Oligonucleotide Purification Cartridges (OPC, Perkin-Elmer ABI) were opened and the resin divided into 45 µl amounts using a micro-titer measuring plate (Millipore). The resin was transferred into a Multiscreen microtiter-filter plate (Millipore) which was placed on a vacuum manifold (Millipore). The resin was preconditioned by adding HPLC grade acetonitrile (50 µl) to each well, followed by 2 M triethylamine acetate (TEAA) (50 µl). The DNA was loaded on the resin by adding 50 µl of PCR product and 50 µl of 0.1 M TEAA. The DNA was washed with an additional 50 µl of 0.1 M TEAA followed by a final 50 µl water rinse. After each addition vacuum was applied. To elute the purified product, 50 µl of 60:40 acetonitrile:water was added to the resin and the eluate collected by centrifugation into another 96-well plate or by removing a 1.5 µl sample directly from each well.

MALDI sample preparation: A 1.5 µl aliquot of each PCR product or standard was placed on a sample well of a 100-well sample holder followed by 1.5 µl of matrix solution (50 mg/ml sublimed 3-hydroxypicolinic acid, 10 mg/ml picolinic acid, and 10 mg/ml ammonium citrate in 50:50 acetonitrile: water). The mixture was allowed to air dry.

Sample acquisition: The spectra were acquired using a PerSeptive Biosystems Voyager DE time-of-flight mass spectrometer. The "autosampler" capability of this instrument allows for automatic acquisition of spectra by moving the laser to a set of user defined coordinates at each sample well. Our method uses twelve positions around the outer perimeter of the sample well. The computer then chooses the best spectra taken for each sample. Each spectrum was the average of 32 laser shots, using a -25KV accelerating voltage, with a 90.5% grid voltage, a 0.150% guide wire voltage and a 300ns delay. After automatic acquisition, samples for which acceptable spectra were not obtained were re-examined manually. Figure 1 shows MALDI spectra of a synthetic oligonucleotide mixture before (A) and after (B) our purification procedure, and a typical OPC-purified PCR product (C) obtained in autosampler mode.

In several trials, the PCR product was detected by MALDI-TOF for each of the 96 samples purified in parallel. In autosampler mode acceptable spectra were obtained for about 90% of the samples. The remaining 10% were subsequently reacquired manually. Resolution (M/ΔM) ranged from <100 to >500, but was typically in the 200-300 range.

It is possible to quickly and effectively purify large numbers of PCR products for MALDI-TOF MS analysis. Using automatic acquisition of spectra by the instrument is an effective way to screen for small PCR products with an appropriate search pattern. The next steps are to look at a statistical analysis of DNA recovery from the purification method and compare autosampler acquired spectra to manually acquired spectra in regards to resolution and signal-to-noise ratio. This will allow for the development of more effective search patterns. We also plan to extend the technology to larger PCR products and mixed-product or multiplexed reactions which may necessitate the development of different matrix preparations. These results represent a significant step toward large scale MALDI-TOF analysis of PCR products in a relatively short time with little user intervention.



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Identification of Methanotrophic Bacteria using the Polymerase Chain Reaction with MALDI-TOF Detection

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Bioremediation is an environmental cleanup strategy that uses microbial action to convert pollutants to less hazardous forms. When bioremediation is performed *in situ* for groundwater decontamination, it is necessary to assess the ability of the microbial population to metabolize targeted pollutants. Conventional methods involve culturing the organisms and measuring in the laboratory their efficiency at degrading specific compounds. More recently, gene probe methods have been developed in which the genes coding for pollutant-degrading enzymes have been identified and can be detected using standard molecular biology techniques such as the polymerase chain reaction (PCR) and hybridization assays. PCR is an enzymatic method for generating many (10^6-10^9) copies of a short segment from an organism's genomic material. Detecting the DNA produced by PCR typically involves a time- or labor-intensive process such as gel electrophoresis.

With the advent of MALDI, however, it is now possible in some cases to detect products of the PCR more rapidly and accurately using mass spectrometry. While typical PCR assays result in oligonucleotides that contain more than 100 bases, MALDI-TOF is at present more suited to detection of DNA species containing less than 100 bases. Therefore, it is useful to design PCR products in this size range.

We have designed and implemented PCR assays to detect the presence of two species of methanotrophic bacteria that are of interest for monitoring bioremediation activity, *Methylosinus trichosporium* OB3b (a type II methanotroph) and *Methylomicrobium albus* BG8 (a type I methanotroph). These assays amplify regions of the *pmoA* gene, which codes for a methane monooxygenase enzyme that can co-metabolize the common industrial pollutant trichloroethylene. The assay for Type I methanotrophs generates a 99-base PCR product, while the type II assay yields a 56-base product. The presence or absence of chromosomal DNA from these two species in the PCR is reflected in the MALDI mass spectrum.

Chromosomal DNA was extracted using standard techniques from methanotrophic cultures grown on agarose plates.[1] Primers were designed to amplify *pmoA* genes from each type of methanotroph but not closely related genes such as ammonia monooxygenase. PCR was performed on a 25 to 100 μL scale using ~200 ng bacterial chromosomal DNA.[1] Following PCR, a rapid (5-minute) reverse-phase purification was performed in microcentrifuge filter tubes using 40-50 mg of resin from an Oligonucleotide Purification Cartridge (OPC, Perkin-Elmer Applied Biosystems). The final elution volume from the reverse-phase purification was 50-100 μL, comparable to the beginning PCR volume. This procedure yielded PCR products of sufficient concentration and purity for subsequent MALDI analysis. Typically, a 1.5 μL aliquot from a single purified 25 μL PCR was mixed on the MALDI sample holder with 1.5 μL of matrix solution (50 mg/mL 3-hydroxypicolinic acid, 10 mg/mL picolinic acid, 10 mg/mL ammonium citrate in 1:1 water:acetonitrile). MALDI-TOF analysis was performed using a PerSeptive Biosystems Voyager-DE in negative-ion mode with delayed extraction and a nitrogen laser.

Figure 1 shows the MALDI spectrum from the PCR product obtained from type II methanotroph DNA, mixed with 5 picomoles of a synthetic DNA 50-mer. The multiple peaks associated with the PCR product arise from the double-stranded nature of the product, non-templated enzymatic addition of an extra base, and the inclusion of inosine bases in the primers, which allows non-specific incorporation of nucleotides in the product. Note the lack of salt adducts or fragmentation in the 50-mer peak, indicating that these phenomena do not contribute to the PCR product spectrum. A "blind" experiment using type II primers with type I, type II, or no DNA showed this 56-mer product only for type II DNA.

The center trace of figure 2 shows the MALDI spectrum of the 99-mer PCR product from type I DNA. The upper two traces in figure 2 are MALDI spectra of synthetic oligonucleotides with sequences corresponding to the two complementary strands of this PCR product, showing slightly better resolution than the double-stranded species. The lower two traces in Figure 2 show that no product was obtained from PCR reactions using type I primers with either type II chromosomal DNA, or no DNA ("water"), demonstrating both the specificity of the primers and the lack of contamination.

To summarize, a single reverse-phase-purified PCR reaction yielded sufficient product to detect chromosomal DNA at M/ΔM ~ 800 for type II (56-mer) or M/ΔM ~ 50 for type I (99-mer) methanotrophs.

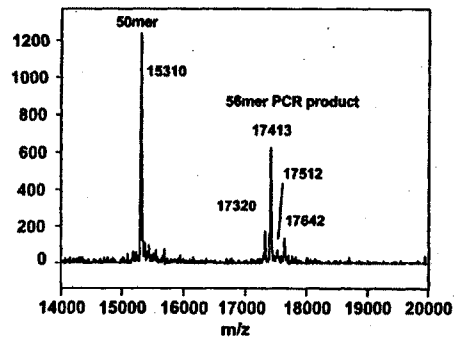

Figure 1

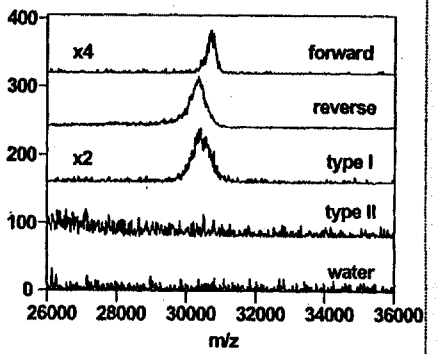

Figure 2

1. 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.* (in press.)

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IMPROVED SPOT HOMOGENEITY FOR DNA MALDI MATRICES

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For size measurement of relatively small DNA molecules such as synthetic oligonucleotides and polymerase chain reaction (PCR) products, MALDI-TOF mass spectrometry [1-5] offers potential advantages of speed, accuracy, and automation over conventional electrophoretic or hybridization techniques. However, with commonly used UV matrices, MALDI of DNA is rather labor intensive for several reasons, one of which is the sparse distribution of "sweet spots" in the final dried sample spot.

In contrast to protein matrices such as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA) and α-cyano-4-hydroxycinnamic acid (CHCA) that yield homogeneous dried spots, well known MALDI matrices for single- and double-stranded DNA such as 3-hydroxypicolinic acid (HPA) and picolinic acid (PA) tend to form the crystals at the rim of their spots. Uneven deposition of DNA-doped matrix crystals on the periphery of the dried spot necessitates a tedious search for sweet spots with the laser. For automated, high throughput MALDI-TOF analysis of short DNA fragments, it is important to obtain homogeneous MALDI spots that yield good signals not only from the periphery but the entire spot.

Several groups [6-8] have shown that substrates such as nitrocellulose, active nafion, and parafilm can be used in order to improve MALDI spot preparation. We have developed a new procedure using polymer substrates and additives to obtain good mass spectra from any location on DNA-doped MALDI spots with a mixed matrix containing HPA and PA. Hydrophilic polymers such as linear polyacrylamide (LPA), poly(ethylene oxide) (PEO), cellulose derivative (methyl cellulose) and other substrates such as Nafion were employed to control the crystal formation of the matrix in order to produce homogeneous spots. Investigation of the DNA distribution in the spot was performed by imaging a synthetic oligonucleotide (20 mer covalently labeled with HEX dye) with a fluorescence microscopy equipped with a CCD camera (see Figure 1).

While DNA/matrix crystals formed only at the spot's rim without any polymer substrate, the use of a combined hydrophilic polymer and Nafion substrate enhanced the homogeneous formation of DNA-doped matrix crystals in the interior of the MALDI spots. Good MALDI spectra were obtained from any region of the spot with high success rate (see Figure 2). Polymer-only or Nafion-only substrate showed limited success, and poor mass spectra were obtained with an anionic polymer (plyacrylic acid, PAA) and a hydrophobic polymer (polydodecyl acrylate, PDA) substrates. We believe that hydrophilic polymer substrates may either reduce transport of the DNA and matrix to the periphery of the spot during the drying process, or influence nucleus formation and growth of HPA and PA crystals. Parameters such as surface tension of the drop, viscosity, molecular weight and hydrophilicity of polymer substrate might also influence crystal formation. Matrix and solvent composition were also studied for the homogeneous spot preparation. Good mass spectra were obtained in the interior of MALDI spot using 50% of acetonitrile with HPA/PA=40:10 mg/mL. Other concentrations of either acetonitrile or HPA/PA did not produce good mass spectra probably due to the different solvent evaporation rate and/or rate of crystal formation.

We are currently working on the application of our spot preparation method to the PCR products (>100 bp) for high-throughput analysis. The goal is to obtain MALDI signal from one location per shot.

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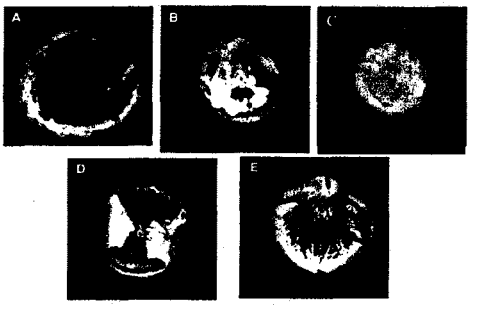
Figure 1

Fluorescence Microscope Images of MALDI spots



A. On gold plate, HPA:PA=4:1, HEX-20 mer DNA,
B. Nafion dried first,
C. LPA dried first, then Nafion dried next,
D. LPA and Nafion mixed together,
E. LPA dried first

Figure 2